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RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES
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BIOCHEMISTRY, COMMUNICABLE DISEASE AND
IMMUNOLOGY, INTERNAL MEDICINE, PHYSIOLOGY,
PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE

Volume II.

(PROJECTS AND WORK UNITS ARE
LISTED IN TABLE OF CONTENTS)

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SUMMARY

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report, and names of the investigators are given at the beginning of each report.

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24. (U) Contemporary epidemiologic methods are applied to causes of disability in military populations. Multidisciplinary collaborative approaches are utilized and new methods developed as required.									
25. (U) 77 10-78 09 Completed are: investigation of the association of nuclear testing to the development of leukemia; determination of the prevalence of antibodies to polio-viruses in recruits; determination of the rate of occurrence of testicular tumors in a command; study of morbidity in Ft. Dix AIT students; injuries during BCT at Ft. Dix; prevalence of leishmaniasis among U.S. troops 1971 to 1977; study of medical processing of recruits; investigation of alleged hypertension in an occupational group; study of morbidity reporting at Ft. Dix. Analyses of the following infectious disease studies are in progress: febrile illness at Ft. Campbell, KY; hepatitis in the 25 Div, HI; tuberculin skin test reactors at Ft. Knox, KY; dengue epidemic in the Bahamas; determination of the prevalence of antibody to adenoviruses in recruits, leishmaniasis among troops undergoing jungle training at Panama; and adenovirus type 21 vaccine trial, Ft. Dix. Analyses of data from the following studies are in progress: determination of congenital malformation rates for several posts; description of renal diseases for active duty personnel; description of the Army's experience with Guillian-Barre syndrome; description of the Army's experience with motorcycle accidents; and system analysis of Ft. Dix General Practice Clinic. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77-30 Sep 78.									

DD FORM 1498

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 001 Epidemiologic Studies of military diseases

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1. Association of Nuclear Testing to the Development of Leukemia

The objectives of the investigation of the association of nuclear weapon testing to the development of leukemia have been completed. These objectives as outlined in last year's Annual Report were:

a. To confirm or reject the 26 possible cases identified by CDC or AFIP as having been present at the test shot and subsequently having developed leukemia.

b. To initiate a broad retrieval of leukemia cases from data bases available to the military to identify cases that might have been at the test shots.

c. To assess the available data and make recommendations for further investigations, if necessary.

Confirmation of the 26 possible cases. A case was confirmed if the individual was present at the test site (his name was on the Smoky list) and he had documented leukemia following the test shot.

Attempts at confirmation of the original case and the nine

possible cases identified by Dr. Caldwell of CDC were done by referral to a list of persons receiving film badges at the time of the test. For confirmation it had to be demonstrated that the social security account number (SSAN) of the possible case was the SSAN that belonged to the individual identified by service number on the Smoky list. The assistance of the National Personnel Records Center and the Veterans Administration was necessary in order to convert service numbers to SSAN's. The diagnosis was confirmed by referral to the military or civilian treatment facility or by VA files.

Diagnosis was already documented for the 16 possible cases identified by the AFIP. The presence of an individual at Test Shot Smoky was confirmed by demonstrating that the identity of a person in the AFIP leukemia file and a person on the Smoky list were the same. Proof of identity was the demonstration that the SSAN or VA claim number of the AFIP case was the same as that assigned to the person on the Smoky list. This was again done by referral to the National Personnel Records Center or the Veterans Administration.

Retrieval of data for additional cases. An attempt was made to identify all cases of leukemia in military hospitals and in central registries available to the military, that might have been at test shot Smoky.

RESULTS

Confirmation of the 26 possible cases. Of the 9 possible cases reported by CDC plus the original case, 5 are not on the Smoky list. One other proved not to have had leukemia. Four were documented as having been at the test and later having developed leukemia.

Of the 16 possible cases from the AFIP files, through linkage of the Smoky list with the military, VA, and civilian sources it was shown that 13 of them had not been present at the test. One other proved to be untraceable. Two were documented as having been at the test.

Retrieval of data for additional cases. The results of the requests for information from various data sources follow:

a. IPDS-IMR - Printed and magnetic tape lists of leukemia cases reported to IPDS from 1963 to the present were received.

The period 1963-1970 used either service number or hospital register numbers as identifiers. The list for 1971-1977 used social security numbers. There were 941 reported cases of leukemia in active duty or retired male Army personnel who were 17 years of age or greater in 1957. The data on hand has been updated and now includes data from 1951 to the present. Resources to convert large numbers of service numbers to social security numbers are not available.

b. CHAMPUS - After an initial agreement to provide information, legal counsel at Office of CHAMPUS determined that such action did not comply with the Privacy Act. No data was sent.

c. Army Medical Center Tumor Registries - All of the tumor registries submitted the requested data. There were a total of 411 leukemia cases reported in active duty or retired male Army personnel who were 17 years of age or greater in 1957. Three of the previously identified cases were found, but no new cases were discovered.

d. Armed Forces Central Medical Registry - This registry sent a list containing 134 cases of leukemia in active duty or retired male Army personnel who would have been 17 years of age or greater in 1957. None of these cases were on the Smoky list.

ASSESSMENT OF THE DATA - Six cases of leukemia have been identified in persons on the Smoky list. Six cases can be compared to a predicted incidence of 2.24 cases with a predicted range due to chance variation of 0.46 to 6.88 cases. The predicted incidence was based upon findings of the Third National Cancer Survey. The predicted range was based on the Poisson distribution with 95 percent confidence limits.

DISCUSSION

There are serious limitations to the data that are presented in this report. Estimates of the population at risk have varied from 2235 to 3143 depending on the criteria that are used to determine presence at the test site. The film badge list (3143) is considered more complete than the morning report figures (2235) but the accuracy of either is not known. Obviously rates based on inaccurate denominator populations can only be rough estimates.

The expected number of cases of leukemia in the group was generated by estimating the ages of those on the list based on

their rank and then applying the Third National Cancer Survey rates to this group. This assumes accuracy of both the age estimation by rank, and extrapolating rates from the general population to a military population.

Perhaps the most serious limitations of the data are the problems of enumerating cases. If the data sources available to the military were complete it is estimated that only 15 to 20 percent of those persons in the military in 1957 would have been treated in a military medical facility at the time they might have developed leukemia and thus be included in the registries and data bases examined. The IPDS-IMR data is not identified by name and thus this relatively large source was not available and further limited the proportion of total cases that would be identified in military records. Of those data sources that were used the individual tumor registries of the Army Medical Center treatment facilities represent the most efficient way to develop data about the cases. Unfortunately the referral policies are not standardized and the registries are incomplete.

Since there is no evidence that the total number of cases of leukemia have been enumerated (the media coverage of CDC, although extensive cannot be considered all inclusive) and the 6 cases identified approach significance, further investigation is necessary.

ADDENDA

Several months after completion of the investigation a search of the VA death benefits claim file by CDC revealed an additional leukemia case who was confirmed as being at Test Shot Smoky.

An additional attempt was made to find new cases by computer matching of service numbers of all leukemia cases in the IMR file, 1963-1970, with service numbers on the roster of Test Shot Smoky. No new cases were found.

The AFIP had generated a list of 47 name matches from a list of 5370 participants at 1951 Nuclear Exercises. It was decided to take a preliminary look at these matches to verify if they had been participants at the test shots. The following steps were taken:

- a. 18 of the 47 cases were found to be errors in matching

and were excluded. One case was a dependent wife of an Air Force member and too young to have been in the Army in 1951 and was excluded.

b. Of the remaining 28 AFIP case records 26 had service numbers which did not match service numbers of participants in the 1951 list (2 of the cases had no service number recorded). Further investigation was undertaken to verify the initial impression that none of the AFIP cases were at the 1951 tests.

c. 14 of the 28 cases had dates of service recorded in VA (Veterans Administration) files, which excluded them (e.g. leaving the service in 1927). VA records also verified three service numbers of cases and the numbers did not match those of the 1951 tests participants.

d. Two cases were civilians who had died from leukemia at civilian hospitals with no record of military service. Although it is suspected that these cases were not at the 1951 tests, it cannot be proven.

e. The nine remaining cases were sent to the National Records Depository at St. Louis, MO. Records on four of the cases show them to have service dates which exclude them as possible participants. There are no records for the last five cases. Those records are thought to have been lost in the fire of 1969.

f. Thus 40 of the 47 AFIP name-matched cases were shown not to have been involved with the 1951 test shots. It is very unlikely that the remaining seven were involved.

Since the 1951 roster was available and we had generated lists of leukemia cases from the Medical Center Tumor files this source of cases was also investigated. There was only one individual contained in the Tumor files that was also on the list of participants.

2. Absence of Antibody to Poliovirus Types in Basic Combat Trainees

This study, which was reported in part in the FY 1977 Walter Reed Army Institute of Research (WRAIR) Annual Report (Work Unit 130, "Viral Infections of Man," and Work Unit 001, "Epidemiologic Studies of Military Diseases"), has been completed. A manuscript dealing with the investigation has been accepted for publication in the scientific literature and is listed at Literature Cited.

3. Determination of the Rate of Occurrence of Testicular Tumors in a Command

This study was reported in the 1976-7 Walter Reed Army Institute of Research Annual Report (Work Unit 001). Following a review of all data, the conclusion stated in the 1976-7 Annual Report (there was no statistically significant increase in the rate of testicular tumor in the subgroup of interest) remains unchanged. All military reports regarding this study have been completed and a manuscript for publication in the scientific literature is in preparation.

4. Study of Morbidity in Fort Dix Advanced Individual Training Students

A study was conducted to look at the medical reasons for Advanced Individual Training (AIT) students missing duty. The study was of cohort design and conducted among the AIT students of the 5th Training Brigade, Fort Dix, NJ, during the period 10 Oct to 4 Dec 1977. One thousand four students were followed during the first 21 days of their training for occurrence of illness requiring hospitalization or quarters. Analysis of illness rates (number of cases per 1000 person days) with the use of the chi square statistic, revealed several trends.

Illness rates were higher for: those who did not receive adenovirus vaccine (16.8) than for those who did receive the vaccine (4.18) ($p < .05$); younger soldiers (6.74) compared to older soldiers (4.14); and, early weeks of training (8.26) compared to latter weeks (4.71). It was also found that women have higher rates for quarters illness (11.15) than men (1.15) ($p < .05$), and, whites have higher rates for respiratory illness (7.22) than non-whites (.94) ($p < .05$). There was no difference in illness rates among different MOS groups.

5. Injuries During Basic Combat Training (BCT) at Fort Dix, New Jersey

In conjunction with preventive medicine residency training, MAJ Carver Wilcox conducted a two week (6-20 March 1978) preliminary study of injuries in Fort Dix basic combat trainees. The study objective was to determine the number and types of orthopedic and podiatric injuries incurred during the seven week BCT cycle. Data collected indicated a trend toward a peak number of injuries occurring during Week #4 and the association of observed injuries with the activities of running and marching. A definitive study may specifically define acti-

vities strongly associated with injuries and thereby identify areas where modification of current training procedures could result in reduced injuries. Such a study should take into account variations in climatic conditions and base line physical fitness levels of trainees.

6. Study of Medical Processing of Recruits at the Fort Dix Reception Center

The U.S. Army Training and Doctrine Command proposed decreasing the time spent by recruits in the Reception Center. Therefore, MAJ Frank Churchill, a preventive medicine resident, conducted a staff study to assess the efficiency of medical inprocessing at the Reception Center in January 1978. After several days of observation it was concluded that medical operations were efficient and no meaningful reduction in the time spent in medical processing could be effected.

7. Investigation of Alleged Hypotension in an Occupational Group

On 16 October 1977, the Preventive Medicine Activity, Fort Dix, New Jersey, was alerted by the Occupational Health Physician of possible hypotension in workers at the Fort Dix refrigeration shop. On 2 and 16 November 1977, CPT Philip Lewis, a preventive medicine resident interviewed employees at the shop and obtained blood pressure readings. Analysis of the blood pressure readings revealed no significant differences from normal systolic ($p > .20$) normal diastolic ($p > .40$), and normal mean pressure ($p > .30$) by Student T tests. Apparent differences were in the direction of hypertension. Analysis of variance revealed significant inter-patient ($p < .005$) differences. Investigation of potentially dangerous substances used by the refrigeration shop showed none to cause hypotension. These facts lead to the conclusion that any clustering of measurements in the low range of normal was probably a chance occurrence.

8. Study of Morbidity Reporting at Fort Dix

In conjunction with preventive medicine residency training, MAJ Frank Churchill studied morbidity reporting by Fort Dix Troop Medical Clinics. He found that the system in existence in February 1978 produced weekly reports which gave only the total sick call attendance and the number of patients with upper respiratory complaints. Additionally, there was no standardized morbidity records keeping system. MAJ Churchill attempted to modify the existing system by establishing standard morbidity reports

which gave numbers of patients seen according to ten different diagnostic categories and disposition data. Although the additional time required to implement the modified system was estimated to be only one man-hour per Troop Medical Clinic per day, the system was rejected by the nursing service and was never fully implemented.

9. Febrile Illness at Fort Campbell, Kentucky

During October 1977 611 soldiers from the 101st Airborne Division (Air Assault), Fort Campbell, Kentucky, participated in jungle training exercises at the Jungle Operations Training Center, Fort Sherman, Canal Zone. About 500 of these men were from the 1/503 Infantry (INF) and the remainder were from a number of other units of the 101st Airborne Division. An advance party (approximately 35 people) departed Fort Campbell on 4 October and the remainder left on 8 October. The entire group returned on 29 October. Medical personnel were not aware of an unusual occurrence of illness in the group during the exercise.

On Friday, 4 November, a physician assistant (PA) saw several patients who had participated in the Panama exercise and who were complaining of fever and headache. He was not impressed by the severity of their illnesses and placed them on quarters. On 7 November he again saw these patients and became concerned that three soldiers with similar febrile illnesses all had been to Panama the preceding month. He consulted a physician and the decision was made to hospitalize all three men. During 8-9 November nine other men, who also had been to Panama during the October exercise, were admitted to the U.S. Army Hospital, Fort Campbell, with similar febrile illnesses. Since a diagnosis could not be established for any of the 12 men and 11 of the 12 were from one small unit of 35 men, the 326 Engineers (EN), Epidemiology Consultant Service (EPICON) assistance was requested.

During 9-12 November a physician from the Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research (WRAIR), conducted a clinical study of 13 hospitalized patients with febrile illness who had participated in the Panama exercise (the one additional patient was admitted on 10 November), and collected specimens for agent isolation and serologic studies. From 10 to 16 November two physicians from the Division of Preventive Medicine, WRAIR, conducted an epidemiologic investigation to:

- (1) Determine if the illness seen in the group of soldiers

who returned from Panama was associated with outbreaks in other Fort Campbell populations.

(2) Determine the extent of illness observed in Fort Campbell troops which might be related to exposures at the jungle training center in Panama.

(3) Identify variables associated with disease.

METHODS AND MATERIALS

The post. Fort Campbell is a U.S. Army Forces Command installation located on the Kentucky-Tennessee border. In mid-November 1977, 21,257 soldiers were assigned to the post; 9,442 dependents lived on the post; 12,459 dependents lived off post; and, 1,714 civilians were employed by the installation. Seventy-nine percent of the military (16,829 soldiers) were assigned to the 101st Airborne Division.

Study of hospitalized cases. Histories were obtained and physical examinations were performed on the 13 admitted patients. Specimens were collected for serologic studies and attempts at agent isolation.

Epidemiologic studies. A questionnaire for physicians and PAs was constructed to determine the incidence of similar illness among troops and civilians at Fort Campbell. Also, PAs accompanying previous Panama exercises were interviewed regarding illness in previous Panama cohorts.

The hospital emergency room log was reviewed for the period 25 September-13 November 1977. All weekend visits and weekday visits occurring from 1600 to 0700 were tabulated for active duty members and for dependents. (Weekday visits between 0701 and 1559 were not tabulated because the emergency room accepted clinic overflow during normal duty hours and variables associated with this procedure were not identified.) Visits for illness were categorized according to disposition diagnoses (respiratory, gastrointestinal, and other).

Attendance logs at five of six 101st Airborne Division troop medical clinics (TMCs) were studied for the period 26 September-11 November 1977. Visits for gastrointestinal disease, upper respiratory infection (URI) - cold, URI - influenza, and respiratory - other were tabulated using disposition diagnoses.

Records from the TMC serving the 1/503 INF were not available, but the log of the battalion aid station used by this unit was reviewed for the period 1 September-14 November (excluding the period the group was in Panama).

During the period 14-22 November all available soldiers who had participated in the October 1977 Panama exercise (270) were requested to complete a questionnaire which asked the following: (1) demographic data; (2) the occurrence of illness during the two weeks prior to going to Panama, while in Panama, and after returning from Panama; and, (3) exposures while in Panama. Soldiers were asked if they were ill. If they responded "yes," they were asked to check their symptoms on a symptom list and to score their symptoms on a scale of 1 to 4 (4 being the most severe). This symptom list was based upon the symptoms observed in the 13 hospitalized patients. Anyone having a score of two or more on any two of the nine symptoms listed was counted as being ill. (This criterion for illness was the minimum criterion which would have identified all 13 hospitalized patients as having been ill).

Serologic survey. In December 1977 serum specimens were obtained from all Fort Campbell troops who had participated in the October 1977 Panama exercise and who were present on the post.

RESULTS

Study of hospitalized cases. Ages, ranks, and units for all 13 hospitalized men, and dates of onset of symptoms and hospitalization for 12 are given in Table 1. Patient 1 presented on 21 October (while in Panama) with a two-day history of fever and stomach pain. He was hospitalized in the Canal Zone and later was admitted to the Fort Campbell hospital (7 November). Twelve of the patients were members of the 326 EN and one was from the 1/503 INF, Company C.

Symptoms for the men from the 326 EN are summarized below:

<u>Symptoms (N=12)</u>	<u>% with specified complaints</u>
Headache	100%
Malaise	100%
Myalgias	92%
Stiff neck	92%
Chills	84%

<u>Symptoms (N=12)</u>	<u>% with specified complaints</u>
Arthralgia	84%
Nausea	67%
Diarrhea	58%
Abdominal cramps	58%
Sore throat	58%
Vomiting	50%
Cough	50%

Physical exam findings for these 12 engineers were: healing insect bites (92%); lymphadenopathy (33%); pharyngitis (33%); and muscle tenderness (25%). Mean maximal temperature in this group (as of the morning of 11 November) was 102°F. Laboratory data were unremarkable except for one man having a questionably abnormal chest film and 33% having at least one abnormal liver function study. Liver function test abnormalities were mild (two had an elevated alkaline phosphatase, two had an elevated LDH, and one had an elevated total bilirubin).

Agent identification and serologic studies. Cultures for bacteria were negative for pathogenic organisms. Several attempts at isolating a virus were unsuccessful. At one time it was thought that a plaque-forming agent was present in some specimens but this was never substantiated. Blood smears were negative for malaria and no ova or parasites were found in stool specimens. Serologic tests for selected rickettsial, leptospiral, and viral agents were negative. Other serologic results suggest the illness observed in most, if not all, of the hospitalized soldiers resulted from infection by *Histoplasma capsulatum*. Analysis of serologic data from hospitalized soldiers and from soldiers who were not ill is still in progress.

Epidemiologic studies. Eighteen of 37 MEDDAC physicians, five of eight 101st Division physicians, and 12 of 15 101st PAs completed and returned the questionnaire regarding the incidence of similar disease at Fort Campbell. Of the 35 responding, four replied "Yes," they had been aware of several patients presenting during a short period with a syndrome similar to that observed in the 13 hospitalized patients prior to 1 November 1977. Twenty-six replied "No" and 5 did not give a "yes" or "no" answer. The "yes" replies are summarized below:

(1) A 101st Division physician referred to what he thought was influenza during the winter of 1976-1977.

Table 1. Ages, ranks, units, and dates of onset of symptoms and hospitalization for Fort Campbell troops who were hospitalized following jungle training in Panama in October 1977.

Patient number	Age (yrs)	Rank	Unit	Date of onset*	Date hospitalized*
1	21	E4	326 EN	+	+
2	36	E7	1/503 INF	30 Oct	9 Nov
3	21	E4	326 EN	1 Nov	8 Nov
4	20	E4	326 EN	2 Nov	7 Nov
5	23	E5	326 EN	2 Nov	7 Nov
6	33	E6	326 EN	3 Nov	8 Nov
7	19	E2	326 EN	4 Nov	8 Nov
8	18	E2	326 EN	5 Nov	8 Nov
9	24	E4	326 EN	5 Nov	8 Nov
10	17	E2	326 EN	6 Nov	8 Nov
11	19	E2	326 EN	6 Nov	9 Nov
12	23	E4	326 EN	7 Nov	8 Nov
13	21	E4	326 EN	7 Nov	10 Nov

*1977.

+ See text.

J.G.

(2) A 101st Division PA mentioned two patients he had seen in the fall of 1977 which were thought to have aseptic meningitis or Rocky Mountain spotted fever.

(3) A MEDDAC pediatrician noted having seen a syndrome similar to that observed in the 13 hospitalized patients in children in early November 1977.

(4) A 101st Division PA referred to some cases he had seen early in November 1977 in a unit which was not engaged in the October Panama exercise. None of these patients were hospitalized.

The review of emergency room records revealed no noteworthy trends or unusual numbers of people seeking care. Review of TMC logs showed that one TMC (TMC #7) had an increase in all respiratory disease in mid-October 1977. Units using this TMC did not participate in the October Panama exercise. No other observations of note were made. Records for the TMC used by 1/503 INF were not reviewed. However, records from the battalion aid station used by this unit (which was staffed by a PA), for the period 1 September-14 November 1977 (excluding the period the group was in Panama), revealed few people from any units in the 1/503 INF presented with illness on any of the days studied.

Two PAs who had accompanied troops to Panama were questioned about the occurrence of illness similar to that observed in the 13 hospitalized patients during prior Panama exercises and the two weeks following the exercises. One PA accompanied a group in November-December 1976. He estimated that four or five men developed a similar illness while in Panama but had no idea what happened after the unit returned to Fort Campbell, since most men went on Christmas leave. The other PA accompanied a group in February-March 1977. He did not think illness similar to that observed in the 13 hospitalized patients was occurring to any degree while his unit was in Panama. He was not aware of an unusual number of people becoming ill after returning to Fort Campbell.

During the October 1977 Panama exercise, corpsmen did not keep sick call logs but the PA accompanying the group did maintain records of patients he had seen (a physician did not accompany the group). Patients with anything more than a minor illness were referred to the PA. The PA's log indicated few people presented with illness during the exercise. Only one

was hospitalized for illness in Panama (patient 1, Table 1).

The occurrence of illness as determined from the questionnaires is summarized in Table 2. Four jungle training exercise units were formed from the Fort Campbell units. In most units in which a high percentage of ill persons was observed, small numbers of people were studied. However, 31 of the 35 men from the 326 EN were studied and 23 (74%) were ill after the exercise according to the questionnaire data.

Review of the schedule of the Panama exercise, as well as interview and questionnaire data, showed that 326 EN personnel had unique experiences while in Panama.

Selected items from the schedule for the Panama exercise are summarized below:

- 8-18 October: Individual training/squad operations.
(Units shown in Table 2 did not operate independently.)
- 19-20 October: 326 EN personnel conducted a civic action project at Fort San Lorenzo (an old Spanish fort). All other units participated in a platoon/company field training exercise.
- 21-27 October: 1st and 2nd squads, 326 EN, operated together as a single independent unit, with only casual contact with personnel from other units. The 3rd squad, 326 EN, accompanied Company C (at least 1/503 Company C and perhaps other elements of Trng. Company C) in field maneuvers.
- 28 October: In garrison in Panama.
- 29 October: Departed for Fort Campbell. Garrison activities resumed.

Specific questionnaire questions regarding exposures for the engineers who were at Fort San Lorenzo showed that individual engineers had different exposures.

DISCUSSION

Data collected and analyzed to date suggest that the febrile

Table 2. Occurrence of illness in Fort Campbell troops before, during, and following an October 1977 jungle training exercise in Panama (questionnaire data).

Exercise unit			% of number studied who were ill:		
Fort Campbell units	Unit Strength	Number Studied	Before the exercise*	During the exercise	After the exercise
Trng. Co. A	180	79	4%	9%	16%
1/503, Co. A	125	47	6%	8%	17%
1/503, CSC	17	8	0%	0%	0%
2/17, Trp. C	20	13	0%	8%	0%
1/503, HHC	4	1	0%	0%	0%
326 MED	4	4	0%	0%	50%
3/319 FA	10	6	0%	67%	50%
Trng. Co. B	182	89	8%	21%	24%
1/503, Co. B	116	53	6%	15%	19%
1/503, HHC	25	7	14%	14%	14%
1/503, CSC	18	10	20%	30%	40%
3rd BDE, HHC	8	7	14%	43%	43%
101 MP Co.	10	7	0%	28%	43%
326 MED	1	1	0%	0%	0%
101 MI Gp.	1	1	0%	100%	0%
553 MP Co.	3	3	0%	33%	0%
Trng. Co. C	182	88	4%	12%	30%
1/503, Co. C	103	48	0%	12%	4%
326 EN	35	31	10%	13%	74%
1/503, CSC	39	8	12%	12%	12%
1/503, HHC	5	1	0%	0%	0%
HOSE	67	14	7%	0%	14%
1/503, HQ Co.	44	6	0%	0%	0%
1/503, Co. A	8	1	100%	0%	0%
1/503, Co. B	8	4	0%	0%	0%
1/503, Co. C	4	1	0%	0%	0%
1/503, CSC	3	2	0%	0%	100%

*Two week period.

J.G.

illness observed in the hospitalized soldiers was due to infection by Histoplasma capsulatum which occurred during jungle training in the Canal Zone. Analysis of serologic and questionnaire data is continuing to determine if infection occurred primarily among engineers and to evaluate relationships between exposures in the Canal Zone and seropositivity.

10. Hepatitis in the 25th Infantry Division, Schofield Barracks, Hawaii

In June 1978 several soldiers from the 25th Infantry Division, who had been admitted to the Tripler Army Medical Center (TAMC) with the diagnosis of hepatitis, were reported to the 25th Division Surgeon's Office. During the period 1 January to 31 May 1978, the Division Surgeon's Office had no cases of hepatitis in the 25th Division reported to them. Therefore, Epidemiology Consultant Service (EPICON) assistance was requested and an EPICON team arrived in Hawaii on 24 June. Prompt definition of the outbreak was essential since a large contingent from the 25th Division (primarily the 1/27 Infantry Battalion) was scheduled to depart for New Zealand on 26-27 June for a training exercise.

During the weekend of 24-25 June, and the morning of 26 June, data were collected and assimilated. By mid-morning of 26 June the following data were available:

- a. Fifteen soldiers with hepatitis (SGOT \geq 80 with no apparent reason for the elevation except viral hepatitis) had been admitted between 28 May and 24 June. Eight had been tested for hepatitis B surface antigen and seven were negative.
- b. All cases were lower ranking (E2 to E5) male, Division soldiers, most of whom were single, lived in Schofield Barracks Quadrangle (Quad) D, and probably ate in the Quad D dining facility.
- c. Nine of the 15 were from the 1/27 Infantry Battalion.
- d. Two cases were in Quad D dining facility workers.
- e. On 26 June, 616 men of the 1/27 had their urine tested by MULTISTIX. Twenty-six (4.2%) had \geq 4 units urobilinogen and/or \geq 2+ bilirubin.

These data were presented to BG Huycke, TAMC Commander, and

members of the TAMC staff on 26 June to aid in formulating a recommendation regarding the deployment of the 1/27 to New Zealand. On the basis of the data given above and the discussion that followed, BG Huycke recommended the unit not be deployed. The Commanding General, 25th Division, accepted the recommendation and the 1/27 was not deployed to New Zealand.

IMMEDIATE ACTIONS TAKEN. In an attempt to stop and/or limit the occurrence of clinical cases, the following actions were taken:

a. On 25 June, because of an apparent outbreak of hepatitis A related to Quad D, administration of immune serum globulin (ISG, 2.0cc) to personnel in units located in Quad D (1/27, 1/35, HHC-1st BDE, and 25 MI) was initiated. This was continued the following week.

b. The Commanding General 25th Division, issued a message to Division personnel which told about the disease, the ways hepatitis virus is transmitted and precautions that should be taken.

c. A water point in the Kahuku Training Area (a Division training area) was found to have considerable coliform bacteria (including mammalian coliforms) on recent testing. The Division Surgeon took steps to insure that water from this water point would be properly treated.

d. All food handlers with hepatitis, suspected of having hepatitis, or who were contacts of a hepatitis case, were removed from the dining facility.

e. Earlier inspections of the Quad D dining facility revealed no significant health hazard with the exception of a dirty ice dispenser. A repeat, extremely thorough inspection by the Division Environmental Science Officer revealed no significant health hazard. (The ice dispenser was removed from the serving line for thorough cleaning.)

THE SEARCH FOR ADDITIONAL HEPATITIS CASES. Four methods were used to identify cases:

a. Known and suspected hepatitis cases reported to the Division Surgeon's Office and/or the TAMC Preventive Medicine Office were followed-up for confirmation. (Confirmation required an SGOT of ≥ 80 mU/ml. Normal was ≤ 40 mU/ml.)

b. TAMC admission and chemistry laboratory data were reviewed daily.

c. In addition to the 1/27, soldiers from the following units also were tested using the urine screen method: 1/35, HHC-1st BDE, 25 MI, and 65th EN. (The 65th EN had supported the 1/27.) Soldiers having a positive urine test were bled for SGOT and bilirubin (SMA 12/60) determinations.

d. Urine screening tests were done on Quad D civilian dining facility workers. Additionally, civilian and military Quad D dining facility workers had SGOT and serum bilirubin determinations done.

NUMBER AND DESCRIPTION OF CASES IDENTIFIED. Twenty-five confirmed cases were identified. Twenty-four were Division soldiers. One was a TAMC soldier who worked on an orthopedic ward, had no association with Schofield Barracks, and had contact with Division soldiers only in the performance of his ward duties. This TAMC case will not be considered further in this report.

Dates of onset of illness and units for the cases are shown in FIGURE 1. The configuration of the curve is consistent with a common source exposure which occurred about mid-May. Five Division units not located in Quad D had one case each (25 S&T, 1/19 CSC, 147 AV, 1/21 C, and 125 SIG). Only one of these individuals could be linked to the Quad D area or personnel in Quad D units. This person from the 25 S&T had two friends in Quad D and ate in the Quad D dining facility. Nineteen cases were from units in Quad D:

<u>UNIT</u>	<u>APPROXIMATE STRENGTH</u>	<u>NUMBER OF CASES</u>	<u>CASES PER 100 SOLDIERS</u>
1/27 HHC	150	1	0.7
1/27 A	170	4	2.4
1/27 B	169	7	4.1
1/27 C	165	0	0.0
1/27 CSC	164	2	1.2
1/35 HHC	172	2	1.2
25 MI	147	2	1.4
HHC 1st BDE	118	1	0.8

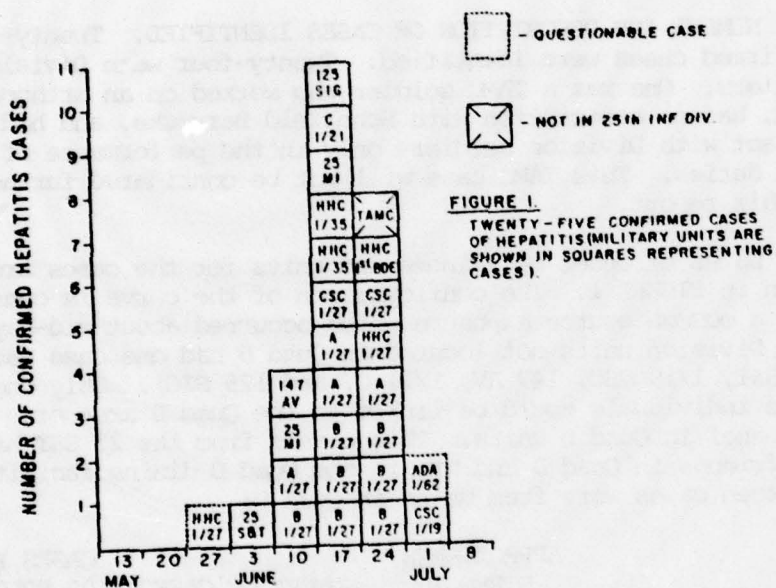


FIGURE 1

TWENTY-FIVE CONFIRMED CASES OF HEPATITIS (MILITARY UNITS ARE SHOWN IN SQUARES REPRESENTING CASES).

WEEK OF ONSET OF SYMPTOMS (WEEK ENDING)
1978

25th INF DIV, SCHOFIELD BARRACKS, HAWAII

JULY 1978

All cases were male soldiers. Ages ranged from 18 to 34 years with a median of 20.5 years. There were two officers (both 1LTs) and 22 enlisted people. Enlisted grades ranged from E2 to E6 with a median of E3. All but one of the 24 were hospitalized. However, as a group, these patients were not severely ill. A summary of clinical symptoms is presented below:

<u>SYMPTOM</u>	<u>% WITH SYMPTOM*</u>
Fatigue/malaise	100
Loss of appetite	88
Dark urine	88
Nausea/vomiting	79
Abdominal pain	79
Headache	79
Fever/chills	75
Yellow skin and/or eyes	71
Light colored stools	54
Diarrhea/loose stools	50
Myalgia	50
Rash/itching	42
Sore throat	33
Arthralgia	21
Cough	17
Constipation	12
*Distaste for cigarettes	77

* Twenty-four patients were studied.

+ Ten of 13 who smoked cigarettes complained of distaste for cigarettes.

FORMULATION AND TESTING OF HYPOTHESES REGARDING THE SOURCE OF THE OUTBREAK. Data obtained from questioning cases suggested that social contacts, unit and other types of parties, and drug usage were not significant factors in this outbreak. Additionally, only seven had had any kind of dental work done since 1 April and only 10 had received any immunizations since 1 April. Patients were also questioned about other types of injections: one had had a TB skin test, one had received a parenteral antibiotic, and one had been given an injected analgesic.

A meeting with the 1/27 commander and executive officer revealed no joint exercises involving multiple 25th Division units during a time appropriate for an incubation period. The only suspicious event was a training exercise in the East Range - Kahuku area during 22-25 May. Only 1/27 Companies A, B, and C,

with a few people from HHC participated. However, only 10 of the 24 cases (42%) recalled ever being in the Kahuku area in May. Additionally, non-ill controls matched by rank and unit, were selected for 16 of the 19 patients from Quad D and questioned. In only five instances did a case report being in the Kahuku area in May when the matched control reported not being there.

Regarding the Quad D dining facility, only 17 of 24 patients (71%) recalled ever eating even a single meal at the facility since mid-April. Only 15 of 24 (62%) reported they usually ate one or more meals (breakfast, lunch, or dinner) at the facility since mid-April. When the 16 cases with controls were compared to their controls, only two cases reported they ate even a single meal when the control did not. Only three cases reported they usually ate one or more meals (breakfast, lunch, or dinner) when the control did not.

Two military dining facility workers were found to have hepatitis. However, their onsets of illness occurred the week ending 17 June, which was too late for either of them to have been the source of the outbreak. No mess hall worker with hepatitis, who might have been the source of the outbreak, was identified.

RECOMMENDATIONS TO DIVISION PERSONNEL.

a. To protect against future cases of hepatitis, strict sanitary standards for garrison and field mess facilities, and strict standards for good personal hygiene should be enforced. Additionally, the provision of properly treated water to troops in the field should be considered of extreme importance. Outbreaks of hepatitis are common in military forces and the events associated with this outbreak provide an example of how hepatitis can severely hinder military operations.

b. Detection of any new outbreak can occur in timely fashion only if cases are reported promptly. This is important since administration of ISG may be of benefit. Division and TAMC reporting procedures should be reviewed and revised if necessary. Additionally, clinical personnel (both in the clinics and on the wards) should be advised of diseases that require reporting and procedures for reporting.

SUMMARY. The source of this outbreak of hepatitis has not been identified. Serologic studies done at the Walter Reed Army

Institute of Research have identified this outbreak as an outbreak of hepatitis A disease. These serologic studies are presented elsewhere on this report (Work Unit 135, "Mechanisms of Transmission of Hepatitis Viruses"). It is hoped that additional serologic studies will better define the case population so that more meaningful comparisons can be made.

11. Tuberculin Skin Test Reactors Fort Knox, Kentucky

The routine school surveillance testing for tuberculosis at Fort Knox was conducted in March 1978. It was noted that one school seemed to have an inordinately large number of convertors. This relatively large number of convertors prompted a request for assistance from the Epidemiology Consultant Service to help determine the focus and extent of the "outbreak".

INTRODUCTION

As part of the routine post surveillance for tuberculosis, yearly tuberculin skin testing is performed on all 1st, 5th, and 9th graders in post schools. This was done March 1978 by the school Health Nurse, using Mono-Vacc Tuberculin tests. Tests were read by the school nurses and positives were referred to the Community Health Nurse's Office for follow-up testing with 5 TU PPD. All PPD tests were read by the same Community Health Nurse.

Only three of the schools (Mudge, Crittenberger, and Van-Voorhis) out of the ten on post had positive reactors in the three grades tested. Mudge Elementary School had considerably more reactors, and a higher rate than the other schools, suggesting that Mudge should be looked at more closely. The previous year (March 1977) a school wide program using Tine Test material had been conducted in all Ft. Knox school children, and based on those results the reactors at this time were considered convertors.

INVESTIGATIONS AT MUDGE SCHOOL

Subsequently all the other children at Mudge Elementary School were tested. The 1st and 5th grades were not retested. Five more reactors were identified for a total of 13 students at Mudge, an overall prevalence of 3.1%.

There were 37 employees at Mudge Elementary School, 32 were skin tested with Mono-Vacc the other 5 were known positives and

were given Chest X-rays, which were read at Ireland Army Hospital, Ft. Knox. Three of the 32 were positive with Mono-Vacc and were still positive on further testing with PPD. None of the known positives or those identified as convertors had evidence of active tuberculosis.

Seventy-seven family members of the 13 children from Mudge (and of 2 children each from Crittenberger and Van-Voorhis Elementary Schools picked up in the initial school screening) were tested with Mono-Vacc and PPD intermediate strength. There were a total of 15 family members positive and of these 7 were identified as convertors. None of the 15 had evidence of active disease.

In-depth interviewing of the originally involved families that were still available on-post, failed to identify a common source or occurrence among the reactors or their families, beside the fact the children attended post schools. Of interest is the fact that at least 7 of these 13 families had an overseas assignment just prior to their tour at Ft. Knox (5 Germany, 2 Panama). The children in the Mudge School ranged in age from 6 to 11 years, from Kindergarten to the 5th Grade suggesting a common occurrence was unlikely.

TESTING OF OTHER POST SCHOOLS

In an effort to determine whether Mudge Elementary School was a major area of transmission, further testing of post schools was recommended. There are a total of 10 schools at Ft. Knox and in 6 of the 10 all students were tested. Three of the seven elementary schools (besides Mudge) and both of the middle schools were tested in June 1978. This testing again consisted of screening with Mono-Vacc and confirming with PPD.

When all grades at these schools (not just the 1st, 5th and 9th grades) are examined, Mudge Elementary School is no longer unique in having a high rate of reactors. Two of the other schools, Stevens Elementary, and Walker Middle School also have high reactor rates. Examination of the children reactors by grade shows the 5th grade has the highest rate but investigation has not revealed a source.

Both the high rate of reactors in children and the large number of staff (3 of 32) that converted indicate transmission at Mudge Elementary School. But the large number of reactors at some of the other schools (Stevens and Walker) and the inability to identify an active case at Mudge School suggests

that transmission is occurring elsewhere. The next obvious area to consider is the school system. It is possible that an active case might have been involved with several of the schools. A substitute teacher, bus driver, parent aide, special teacher, etc., that worked in more than one school and more than one classroom could explain the distribution of reactors. These individuals are being identified and tested. There are a large number of substitute teachers and most of them taught in more than one school.

The seventy-seven family members of the 17 children originally identified as convertors included 15 positive reactors and 7 of these were considered convertors. Only one of these 7 had any connection with the school system. This suggests transmission occurring outside the schools.

EXAMINATION OF OTHER DATA SOURCES

No screening comparable to the program in the schools has been done among other groups on post to indicate that the problem is not more widely spread. In an attempt to obtain data on tuberculin reactions among other groups on post, records were reviewed that related routine testing for tuberculosis. Unfortunately these data sources were of little value since there was no standardized method of recording results. The recruit immunization program maintains the best records but changes in the epidemiology of tuberculosis at Ft. Knox will not be reflected in this group.

HOUSING AREA INVESTIGATION

Spot maps were made of the place of residence of all 48 school children reactors identified. As would be expected, the housing areas serving schools with high rates had concentrations of reactors. If transmission has to be explained outside the school system, then this housing data may be helpful. The considerable discrepancy between the two areas of the VanVoorhis Manor housing complex is intriguing (Table 3).

TABLE 3: Housing Area For Positive Reactors in the Schools*

<u>Housing Area</u>	<u>Rank</u>	<u># Units</u>	<u># Bedrooms</u>	<u># Reactors</u>	<u>Prevalence/ 1000 Bedrm</u>
VanVoorhis-S. of Chaffe	E6-7	616	1764	15	8.5
VanVoorhis-N. of Chaffe	01-03	1096	3126	5	1.6

TABLE 3: (continued)

<u>Housing Area</u>	<u>Rank</u>	<u># Units</u>	<u># Bedrooms</u>	<u># Reactors</u>	<u>Prev/ 1000 Bedrm</u>
Rose Terrace	E4-5	546	1326	6	4.5
Pritchard	E4-5	420	1080	9	8.3
Gaffey Hts.	E4-5	348	822	3	3.6
		3026	8118	38 [#]	4.6

* This includes only those housing areas where all the students were tested.

[#] The 10 other children were scattered in the other housing areas.

Table 3 shows the number of reactors identified in the schools by the housing area in which they live. Since the denominator for number of children tested in these areas is not known, for comparison, a rate based on number of bedrooms in the housing area complex was used. There are obvious potential difficulties with this comparison, in that the relationship of ages and numbers of children to numbers of bedrooms may vary by housing area. But, using these rates based on bedrooms, one housing area (North VanVoorhis Manor) had a rate significantly lower than the other areas.

DISCUSSION

Any conclusions must be based on two major assumptions: (1) that a positive skin reaction indicates past infection with Mycobacterium tuberculosis and (2) that those considered converters indeed represent recent infection occurring at Ft. Knox.

Atypical mycobacterial disease is present in Kentucky, and with minimal skin test reactions there is the possibility that at least some of the converters are exhibiting sensitization to atypical mycobacteria. The larger the reaction the less likely that this is occurring, but a number of the converters did have relatively small reactions.

BCG vaccination is another possible complicating factor. A number of the children were born overseas in areas where BCG vaccination might have been used, and the historical data is of questionable accuracy. It is known that BCG vaccination converts the skin test reaction, but the size of the reaction decreases with time. There is also recent evidence that BCG given before one year of age will not cause reactions larger than 10 mm later in life. The effect of BCG on the reactors in the schools should be negligible since they are considered convertors and thus must have had a recent negative reading and the present positive would be indicative of recent infection. But, it is possible that they were not tested, or were considered negative on the previous screening because of the history of BCG vaccination.

Recent conversion for the most part is based on the comparison of the tine test program the previous year and the results of the Mono-Vacc screening this year. It is accepted that the Mono-Vacc test is more sensitive than the tine test, and a number of these new reactors might have been positive a year ago if they had been tested at that time with Mono-Vacc material, i.e. it is possible that some of these were false negatives a year ago and thus their exposure occurred previous to the tine test program. Unfortunately, immunization records and parents reporting of results also may frequently be in error. The records frequently indicate that a test was applied but there is no indication of the result. In most of these cases the parent is queried whether the test was positive or negative and their response is then entered on the record. It is thus likely that some of these recent convertors had converted previous to these screenings and were likely infected at a previous assignment. The frequent overseas experience of this group makes previous exposure that much more likely.

The problems of interpretation concerning the three points above, atypical disease, BCG vaccination, and sensitivity and specificity of the test and the recorded result, all emphasize the lack of good basic background data on prevalence of tuberculin positivity in U.S. military dependent population.

With the qualification that a number of those considered convertors may not be such, there are still enough "real" convertors to be worrisome, and this is indicated in the difference in testing results between school year 1975-76 and 1977-78 when Mono-Vacc was used both years. Repeat testing of the school population this fall with the Mono-Vacc screening test

will allow a much better estimation of the amount of exposure and conversion presently occurring in this population.

The limitations in the interpretation of a positive test make it impossible to delineate the extent of the present "outbreak." If all of the school children reactors are considered recent converters then the first task is to rule-out transmission in the schools. If this can be done then the analysis of the housing area takes on more credence. Admittedly the comparison of housing areas using the prevalence by bedrooms assumes consistency between the number of children and the number of bedrooms in the various housing areas but, if transmission is ruled out as occurring in the school system, then the low rate in the North VanVoorhis Manor housing area is substantiated by the low rate in VanVoorhis Elementary School that serves this housing area. This is in contrast to the rates of the other schools reflecting increased transmission in the housing areas they serve.

Assuming that this analysis is correct, that the large housing area of North VanVoorhis has low rates of transmission, this indicates that transmission is not taking place in areas that should be frequented equally by persons in the different housing areas. Thus, the Commissary, the PX, recreation areas, restaurants, etc., would be unlikely areas of transmission.

Unfortunately, all schools on post were not completely screened so that the extrapolation from the schools to the housing areas can only be made in some of the housing areas. In Table 3 it is apparent that North VanVoorhis is different from the other housing areas because it houses officer personnel. There are several reasons why this may relate to the epidemiology of tuberculosis. It is well known that the prevalence of tuberculosis is related to the socio-economic level of the population. In the military community, an obvious division of socio-economic levels is by rank. This difference may or may not be related to the present situation at Ft. Knox. Another possibility may be the density of foreign-born wives in the different housing areas. The disproportionate representation of foreign-born wives on active case files (9 of 10 at Ft. Knox) and on chest clinic follow-up files (50% of dependent wives at Ft. Knox) indicates the potential importance of this group in the epidemiology of tuberculosis on military reservations.

Testing of all persons involved with the schools will be

repeated in October 1978. Results of the retest will provide a better estimation of the present situation.

12. Dengue Fever Outbreak in the Bahamas 1977

A pandemic of Dengue fever swept through the Caribbean during calendar year 1977. WRAIR was directly involved with laboratory and epidemiological consultation in Jamaica during July 1977 (see WRAIR Annual Report 1977) and in the Bahamas during November of 1977. The following is a discussion of the investigation in the Bahamas, information on the laboratory aspects of the investigation are reported under Work Unit 130, "Viral Diseases in Man."

THE OUTBREAK

Early indications. Shortly after the official reports on the occurrence of dengue in Jamaica in July, 1977 the medical profession and health services in the Bahamas were alerted on the need for increased surveillance with submission of paired sera from the sporadic cases of influenza that were already being reported. Partly as a result, it became evident that cases of dengue had occurred in New Providence as early as the first week of August, 1977. The first was a Bahamian who went to Jamaica on 25 July, had onset of symptoms on 4 August, returned home on the 7th and consulted a private practitioner on 8 August. The acute and convalescent sera yielded dengue HI titers of 1/40 and 1/1280 respectively. The second, a resident of Nassau with no history of recent travel out of the Bahamas had a history of onset on 5 August with signs and symptoms identified by his nurse-educator spouse as typical of dengue. He volunteered for blood collections on 16 and 26 August both of which yielded HI titers of 1/640. Because of unnecessary delays in referral of the specimens and receipt of the reports, these positive results became available only on 24 October.

Meanwhile, there had been a steady increase in the weekly reported number of cases of influenza, popularly named "Jamaica Flu." Although an occasional case of dengue was reported, it was not until the week of 16 October that any appreciable numbers of that disease appeared in the returns.

Investigations. With the confirmation of transmission in New Providence, medical practitioners were specifically alerted on the possibility of Dengue and PAHO's assistance was sought to study the situation.

As a result, two epidemiologists from WRAIR and CDC, an ecologist/vector control specialist from Grand Cayman and a laboratory technologist from Caribbean Epidemiology Center arrived in Nassau during 6-7 November. With the collaboration of national personnel, a series of investigations was carried out.

Review of weekly communicable disease reports. For purposes of comparison, the reports of influenza and dengue in New Providence were compiled for the epidemiological weeks 27 June-26 December, 1976 (when no dengue had occurred) and 26 June-25 December, 1977 (Table 4 and Fig.2). The totals recorded were 687 influenza in 1976 and 2562 combined influenza/dengue (including 1054 dengue) in 1977.

Data on sex and age distribution available for 2039 of the 1977 cases showed that the syndrome affected males (1058) and females (980) equally, but predominantly (75.2%) the age group 15-44 years. The under 15's accounted for 14.2% and those 45 years and over 10.6% of the cases.

From the Family Islands, 96 cases of combined influenza/dengue were reported during the 13-week period commencing 2 October: Grand Bahama 45 (of which 41 were from West End), Eleuthera 35, Andros 28, Inagua 5, Long Island 2, and Exuma 1 (a visitor from Nassau).

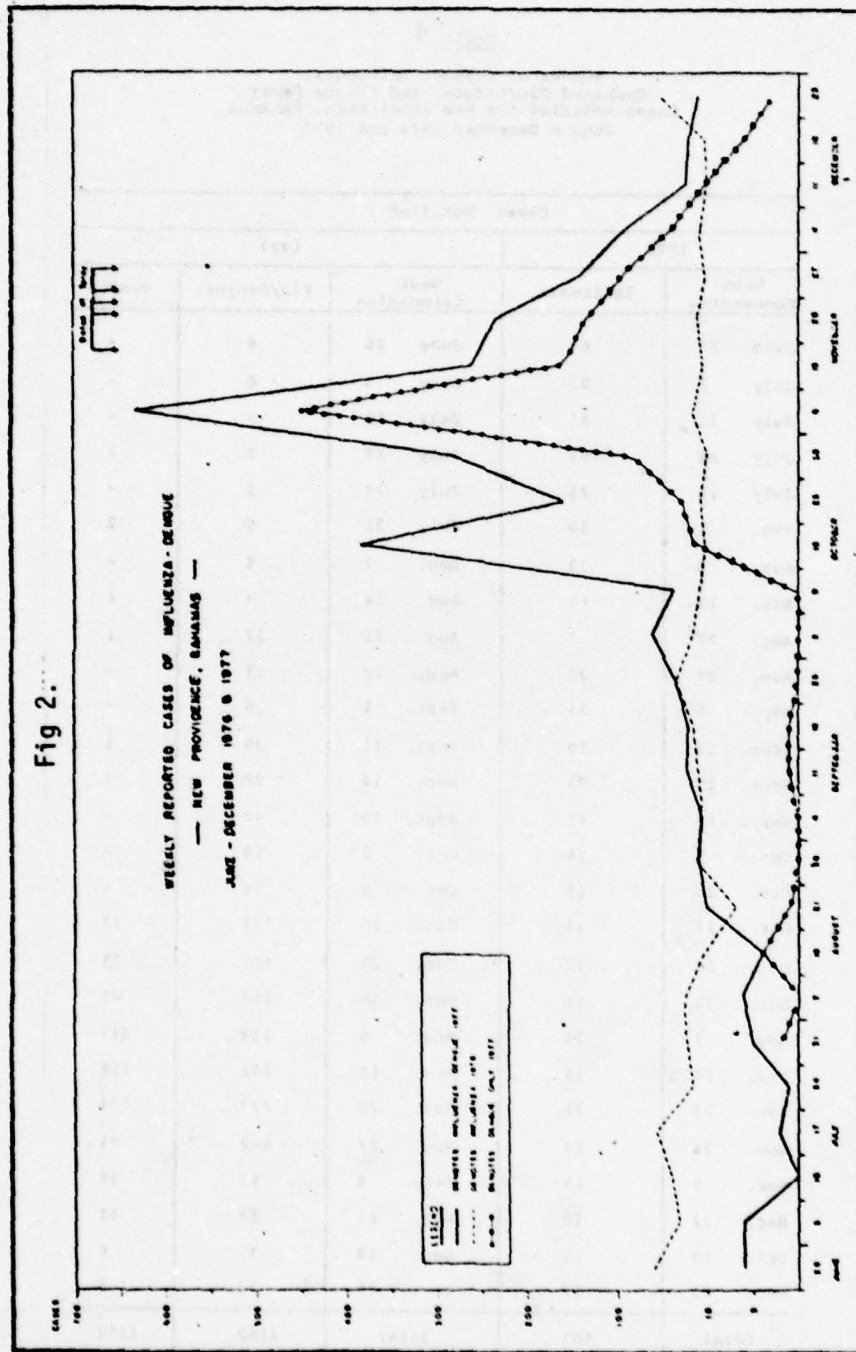
Clinical complex. During the week of 6 November an epidemiological questionnaire incorporating 22 items comprising the clinical complex of suspected dengue fever was filled out at the Outpatient Service of the Princess Margaret Hospital (PMH) in Nassau. The results of replies given by 131 patients yielded a clinical complex of fever (97%), headache (94%), retro-orbital pain (86%), chills (85%), weakness (82%), backache (75%), dizziness (75%), muscular pain (72%), and joint pains (63%). Twenty to 27% of cases reported coryza, cough or sore-throat but 13 throat washings examined at CDC were negative for influenza virus. Seventeen percent reported a skin rash while 4-5% acknowledged to mild haemorrhagic signs.

Geographical distribution. It was possible to obtain details on the address of only 34 cases during the early weeks, but a concerted effort during 11 November-1 December at the Princess Margaret Hospital Outpatient Service provided this information for an additional 198. These are all virtually limited to the eastern half of the island where the population is centered.

TABLE 4
Number of Suspect Influenza,
Combined Flu/Dengue, and Dengue Fever
Cases Notified for New Providence, Bahamas
June - December 1976 and 1977

Cases Notified				
1976		1977		
Week Commencing	Influenza	Week Commencing	Flu/Dengue	Dengue
June 27	63	June 26	6	-
July 3	29	July 3	6	-
July 11	45	July 10	-	-
July 18	61	July 17	2	-
July 25	25	July 24	1	-
Aug. 1	30	July 31	5	2
Aug. 8	33	Aug. 7	6	-
Aug. 15	15	Aug. 14	4	4
Aug. 22	7	Aug. 21	12	1
Aug. 29	20	Aug. 28	19	-
Sept. 5	15	Sept. 4	16	-
Sept. 12	19	Sept. 11	30	1
Sept. 19	23	Sept. 18	28	1
Sept. 26	41	Sept. 25	42	-
Oct. 3	26	Oct. 2	66	-
Oct. 10	19	Oct. 9	36	-
Oct. 17	13	Oct. 16	385	23
Oct. 24	17	Oct. 23	160	35
Oct. 31	11	Oct. 30	284	83
Nov. 7	25	Nov. 6	634	449
Nov. 14	16	Nov. 13	261	159
Nov. 21	21	Nov. 20	237	134
Nov. 28	13	Nov. 27	142	94
Dec. 5	19	Dec. 4	92	46
Dec. 12	11	Dec. 11	33	13
Dec. 19	13	Dec. 18	32	6
Dec. 25	57	Dec. 25	23	3
Total	687	Total	2562	1054

Fig 2.



Attack rate. A spot survey of 20 households in each of seven districts of Nassau attempted by interview to establish attack rates for dengue-like syndrome. The survey covered 139 houses with 712 residents and yielded an overall attack rate of 11.8% varying between 4.5% in Donottage and 28% in Grove - both middle class areas - with rates of 15.5% and 12.2% in Englerston and Oakesfield respectively in the more densely populated sectors.

The first two Family Islands from which suspected dengue was reported were visited briefly. At Matthews Town, Inagua interview of 93 residents in 17 households gave six positive replies to queries on signs and symptoms of dengue fever, but 77 receptacles - including inadequately protected cisterns, water drums and old car tires - in 31 premises were all negative for Aedes aegypti. In Georgetown, Exuma, three of 75 residents in 17 households also gave positive replies to such queries, but 20 containers in 24 premises showed only one tire with larvae.

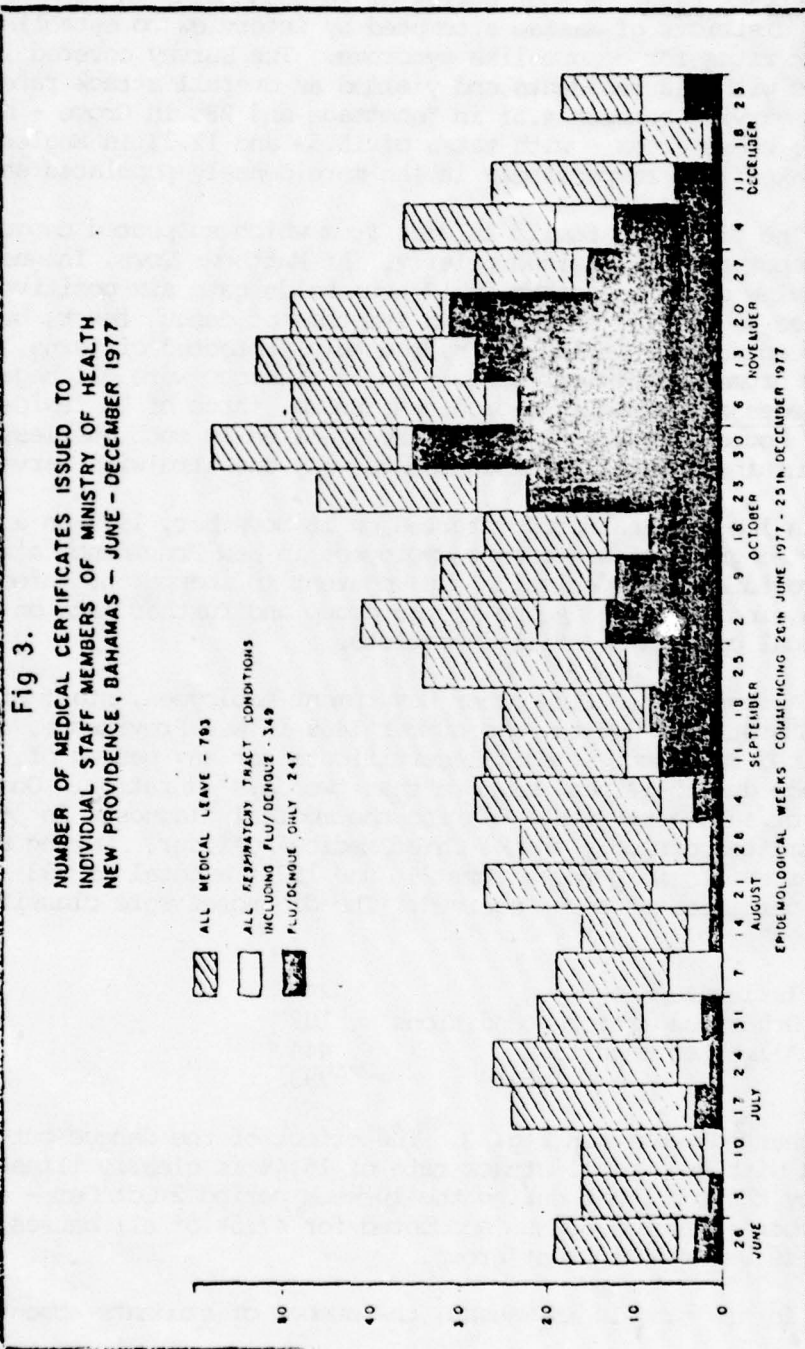
A longitudinal study started on 18 November, 1977 on a selected group of government employees in New Providence aimed at providing information on the apparent to inapparent infection ratio, incidence of dengue in the group and further data on clinical complex is still incomplete.

Absenteeism. Like other Government employees, staff of the Ministry of Health, who number 1469 in New Providence, are required to submit a medical certificate for any period of absence due to illness of more than two days' duration. One part of each certificate bearing the medical diagnosis is transmitted directly to the Chief Medical Officer. During the 27 epidemiological weeks from 26 June 1977, a total of 793 such certificates were received. The diagnoses were classified as -

Influenza/dengue	247
Other respiratory conditions	102
All other conditions	444
Total	793

and charted weekly in Fig. 3. The effect of the dengue outbreak with an overall attack rate of 16.4% is clearly illustrated by the fact that during the 10-week period 2 October - 4 December influenza/dengue accounted for 47.5% of all causes of illness reported in that group.

In six schools in Nassau, the number of students absent



was recorded by first day of absenteeism in weekly intervals from the first week of the Autumn Term starting 5 September 1977. There was a gradual rise from eight to seventeen by the week of 16 October, followed by a sharp increase to 53 coinciding with the peak week of the dengue epidemic of 6 November, 1977.

Laboratory Diagnosis. Paired sera of suspected dengue cases referred either by the Outpatient Service or by private practitioners were sent for diagnosis either to CAREC in Port-of-Spain or the Microbiological Department, University of the West Indies, Jamaica. On 126 results received for New Providence, a total of 69 or 54.8% were positive for Group B Arbovirus, interpreted in the Bahamas as dengue fever.

On the other hand, in an attempt to isolate the dengue virus, 76 sera collected from patients attending the Outpatients Service in Nassau within 2-3 days of onset of typical symptoms of dengue during the week of 6 November were inoculated directly into mosquito cell cultures, using the cell line LSTM-AP-61 and incubated at room temperature. Aliquots were shipped to Walter Reed Army Institute of Research (WRAIR) for similar attempts. At CAREC, 51 (67.4%) developed cytopathic effects identical to that produced by dengue virus, of which nine were subsequently identified as Dengue Type I. At WRAIR, five of the isolates had also been similarly identified by 15 December, 1977.

The Vector. In view of the low house indices recorded by the Vector Control Unit (4%) and in the face of the indications of dengue activity from mid-August, a rapid assessment of the degree and distribution of *Aedes aegypti* was undertaken during November 7-9 to determine the adequacy of its vectorial potential to support an epidemic. This was carried out by a spot sampling survey of premises along transects across the city of Nassau and the remainder of the island, with a higher sampling rate in the areas of densest population, and including some of the ubiquitous collections of roadside garbage and garage tire dumps. In each location, the search was discontinued after finding a positive focus. Of a total of 100 premises and localities thus inspected, 62% were found infested principally in the eastern half of the island but with isolated foci in Gambier, Adelaide and Coral Harbour in the west. In Greater Nassau, a densely populated Sector had an index reaching 82.8%. One of the features which characterized *Aedes aegypti* breeding was the opportunity offered by garbage-littered and weed-grown vacant building lots for the mosquito to spread its range by moving from container to container.

In anticipation of vigorous source reduction from a clean-up campaign 500 oviposits were distributed on 10 November throughout New Providence for subsequent evaluation of breeding activity. When it became evident that that expectation would not be realized, the oviposits were removed during the two weeks ending 17 December, in the course of which 12.9% of 310 examined were positive for Aedes aegypti.

CONTROL

Because of very limited local resources in trained manpower, equipment and suitable supplies, the choice of methods for prompt interruption of transmission of dengue rested in airsprays. Approval for this and emergency funds was granted by Cabinet on 8 November and contractual arrangements were made with a US company experienced in such operations. Malathion 95% concentrate was used at a standard dosage of three fluid ounces per acre (equivalent to 0.222 lb/acre or 249 gm/hectar active ingredient). The aircraft was a DC3, converted for ULV spraying, fitted with booms and No. 8004 nozzles flown at approximately 150 feet altitude to deliver an effective swathe of 1,000 ft. downwind from each flight line. Each swathe's position was left to the judgment of the experienced airspray pilot, guided by clearly marked maps of the area.

The areas to be treated were selected primarily on the basis of levels of Aedes aegypti infestation as revealed by the spot survey of 7-9 November as well as the topography and other features of the terrain recognizable to the pilot. A total of 24,227 acres were treated, including 7,404 acres of Nassau City which had the highest indices and was marked for one additional spraying.

The data compiled illustrated clearly the occurrence of an epidemic of Dengue Type I in New Providence which started with sporadic cases during late July-early August 1977, grew gradually during August to mid-October when it was not fully identified clinically, apparently peaked by mid-November and faded out by the end of the year. This is amply substantiated by the records of absenteeism at six schools in Nassau, as well as that among the representative group of adult employees at the Ministry of Health. The virus was presumably introduced from one or other of the Caribbean countries experiencing outbreaks of their own. With the vector widely prevalent at high levels of infestation, the stage was well set for the outbreak.

13. Prevalence of Antibody to Seven Adenovirus Types in Basic Combat Trainees

This study was reported in part in the FY 1977 WRAIR Annual Report (Work Unit 001, "Epidemiologic Studies of Military Diseases"). Analysis of data is continuing, with associations between seropositivity and demographic variables being investigated.

14. Simultaneous Administration of Live, Enteric-Coated, Oral Adenovirus Types 4, 7, and 21 Vaccines: Safety, Efficacy, and Immunogenicity

This vaccine trial was conducted at Fort Dix, New Jersey, during the period October-December 1976 and was reported in part in the FY 1977 WRAIR Annual Report (Work Unit 001, "Epidemiologic Studies of Military Diseases"). Completion of work on this study was hampered by a lack of primary human embryonic kidney cell cultures for neutralizing antibody tests. During FY 1978 alternate serologic tests were investigated and are reported elsewhere (Work Unit 130, "Viral Infections of Man").

15. Description of Renal Diseases for Active Duty Personnel

The principal investigator for this study (LTC Hodder) was transferred to the Uniformed Services University of the Health Sciences (USUHS) School of Medicine. The study is being continued at USUHS.

16. Morbidity and Mortality Resulting from Motorcycle Accidents in the U.S. Army

This project is designed to determine the cost of motorcycle accidents to the U.S. Army. Using data that have been generated on inpatients by the IPDS system an evaluation of the cost, in time lost from duty, disability and deaths will be attempted.

Tapes from the IPDS system have been obtained that cover the years 1973-1978 coded for injuries resulting from motor vehicle accidents. From these tapes it will be possible to determine the numbers of accidents, the types of injuries that resulted and a basic demographic breakdown of the victims. The data presently is in the form of 300 character records on computer tape from the Patient Administration Systems and Biostatistic Activity at Ft. Sam Houston, Texas. The data will be formatted and retrieved in tabular form. If the initial

analysis of the data supports the suspicion that indeed motorcycle accidents account for significant medical costs to the Army, then further analysis and studies will be planned.

17. Prevalence of Leishmaniasis in U.S. Army Troops (1971-1977)

Because of growing concern over the importance of leishmaniasis as a disease threat to large numbers of soldiers undergoing jungle training in the Canal Zone, MAJ Henry M. Scagliola, a preventive medicine resident, undertook a study to determine the numbers of hospitalizations which have occurred over recent years due to this disease. The study established the number of initial hospitalizations which occurred in active duty U.S. Army personnel between 1 January 1971 and 30 June 1977. Initial hospitalizations were chosen to identify cases because the accepted mode of treatment (antimonial compounds) necessitates hospitalization of the patient during treatment.

METHODS

Data were gathered from one primary source and three secondary sources. The primary source chosen was the Individual Patient Data System (IPDS) of the U.S. Army Health and Data Systems Agency. IPDS is a computer oriented collection of medical and demographic data for in-patients discharged by the Army Medical Department. Data are received from Army Hospitals worldwide, on a weekly basis in the form of magnetic tapes, punched cards, clinical record cover sheets, and coding transcripts. Records are placed on tape and processed for the preparation of monthly, quarterly, semiannual, and annual reports. Records for individuals with specific diseases are retrievable using International Classification of Diseases (Adapted) code numbers. A request for all cases of cutaneous and mucocutaneous leishmaniasis was submitted to the Office of the Army Surgeon General and a magnetic tape containing the requested records was forwarded to the Walter Reed Army Institute of Research, Division of Biometrics.

To provide a check of the IPDS reported cases, three secondary sources were utilized:

a. Through the cooperation of the Infectious Disease Consultant for the Office of the Surgeon General, all Med-16 Surveillance reports for leishmaniasis during the specified time period were obtained. Under AR 400-418 leishmaniasis is a reportable disease, and therefore, theoretically all cases which

occurred should have been reported by Med-16.

b. Through the cooperation of MAJ Hendricks, Division of Medicinal Chemistry, Walter Reed Army Institute of Research, the treatment file of the U.S. Army Medical Research Unit/Panama for all patients hospitalized at Gorgas Hospital for cutaneous Leishmaniasis was obtained. This was chosen because all patients diagnosed and treated at Gorgas Hospital who were in the U.S. Army would be listed.

c. Finally, the Department of Geographic Pathology of the Armed Forces Institute of Pathology was requested to supply information concerning all specimens referred to it which were given the pathological diagnosis of cutaneous Leishmaniasis.

RESULTS

For the period 1 January 1971 to 30 June 1977, 113 initial hospitalizations for leishmaniasis were identified in active duty Army personnel. The majority (at least 89) were at Gorgas Hospital (Canal Zone). Twelve occurred elsewhere at Army treatment facilities in CONUS. Two were at treatment facilities overseas (not the Canal Zone) and the remaining ten locations could not be determined. Initial hospitalizations by location and year are given below:

<u>Year</u>	<u>Location</u>		<u>Total</u>
	<u>Canal Zone</u>	<u>Unknown and Elsewhere</u>	
1971	42	6	48
1972	14	4	18
1973	12	0	12
1974	8	3	11
1975	1	0	1
1976	2	5	7
1977*	10	6	16
TOTAL	89	24	113

* First six months only.

The majority of initial hospitalizations took place before 1973. This is thought to reflect the high number of personnel exposed to jungle training in the Canal Zone because of the Vietnam conflict.

The mean age of patients was 24.4 (median of 22). Seventy-six percent were white, 24% were black. All of the patients were male. The frequency of re-hospitalization for leishmaniasis was determined. Approximately 80% of the cases were hospitalized once, whereas 15.9 were hospitalized twice, 2.7% three times, and one patient required five separate hospitalizations. Re-hospitalization probably reflects initial treatment failure with reoccurrence of disease.

Sixty-one percent of cases were reported by IPDS, 19% by Med-16, 79% by the Gorgas Hospital file and 3% by the Armed Forces Institute of Pathology, Division of Geographic Pathology. In order to determine the completeness of the IPDS data base, the percent of all cases which were listed by the IPDS was determined. As can be seen in the table below, when the IPDS system was first initiated in 1971 its completeness was low, however its completeness has increased until the present when it appears to have on record all known cases.

<u>Year</u>	<u>IPDS Reported</u>	<u>Total Reported</u>	<u>% Complete</u>
1971	18	48	38%
1972	10	18	56%
1973	7	12	58%
1974	8	11	73%
1975	1	1	100%
1976	7	7	100%
1977	16	16	100%

DISCUSSION

From 1 January 1971 to mid-1977, 113 known cases of cutaneous Leishmaniasis were hospitalized and treated at Army treatment facilities (none of the reported cases were visceral or

mucocutaneous forms of the disease). Seventy-nine percent of the cases were known to have been treated in Panama. The remaining 21% were treated elsewhere or the place of treatment was unknown. The place of origin of these infections could not be determined from the data obtained.

Although the IPDS reporting system was incomplete for 1971-4, more recent data suggest the IPDS system is currently an acceptable data base for identifying cases of cutaneous Leishmaniasis.

18. Leishmaniasis Among Troops Undergoing Jungle Training in Panama. In the summer of 1978 MAJ E. Takafuji (Preventive Medicine Officer, Fort Bragg, North Carolina) requested that the Walter Reed Army Institute of Research (WRAIR) assist him in conducting a prospective study of approximately 600 soldiers. The soldiers were to be deployed to the Canal Zone later in the year for about three weeks for jungle warfare training. The objectives of the study were to identify soldiers who had developed leishmaniasis and to establish the incidence of leishmaniasis in the study population. WRAIR provided professional, technical, and laboratory support. Individuals from the Divisions of Communicable Disease and Immunology (COL C. Diggs), Experimental Therapeutics (MAJ L. Hendricks), and Preventive Medicine (MAJs K. M. McNeill and H. M. Scagliola) participated.

METHODS

Prior to deployment, study subjects were questioned about past exposure to leishmania endemic areas, had serum specimens drawn, and underwent examinations to identify and characterize skin lesions. At six weeks and at five months after returning from the Canal Zone, serum specimens were again drawn and examinations were again conducted.

Serum specimens were tested using an immunofluorescent immunoglobulin technique. Suspicious skin lesions were cultured for the presence of leishmania organisms.

RESULTS

The serologic test lacked both sensitivity and specificity. Eleven culture confirmed cases of leishmaniasis were identified following jungle training, for an overall incidence of approximately 1.8/100 soldiers. However, in one company the incidence exceeded 4/100 soldiers. A manuscript dealing with this study is currently in preparation.

This study is complementary to work described under Work Unit 083, entitled "Protective Immunization in Protozoan Diseases," and Work Unit 099, entitled "Chemotherapy and Chemoprophylaxis of Leishmaniasis."

19. System Analysis of a Fort Dix General Practice Clinic.

During the period 19-30 June 1978, MAJ H. M. Scagliola conducted a trial surveillance system at a Fort Dix, New Jersey, outpatient clinic. Using a questionnaire which was filled in by both the patient and the physician, data on the following were collected for 535 patients: age, sex, race, rank, status (retired, dependent, military trainee, etc.), source of referral, chief complaint, diagnosis, and disposition. At the end of the trial period it was concluded that the surveillance system could be implemented with minimal difficulty. Additionally, data collected identified areas where modification of existing methods for patient assessment and referral might improve the efficiency of the medical care system.

20. Determination of Congenital Malformation Rates for Seven U.S. Army Posts.

Congenital malformation rates at seven installations for the period 1 January 1971 through 30 June 1975 were studied using Individual Patient Data System (IPDS) computerized hospital discharge records. The events leading up to this study and the methodology are described in the 1976-77 Walter Reed Army Institute of Research Annual Report (Work Unit 001, entitled "Epidemiologic Studies of Military Diseases").

Data collection for this project has been completed and the final report is in preparation. Figures 4, 5 and 6 give liveborn infants with congenital anomalies/10,000 livebirths for Category I, Category II, and Category III congenital anomalies, respectively. The actual numbers of infants with anomalies are shown in the parentheses. (Category I anomalies are of little or no medical or cosmetic significance. The nature of Category II anomalies could not be determined. Category III contains anomalies which are considered major malformations, are likely to be correctly diagnosed by a general pediatrician, and are likely to be coded correctly). The most striking observation is the consistent upward trend noted for Fort Campbell.

The study presented here was intended as a rapid, preliminary study of congenital anomalies. After a thorough review

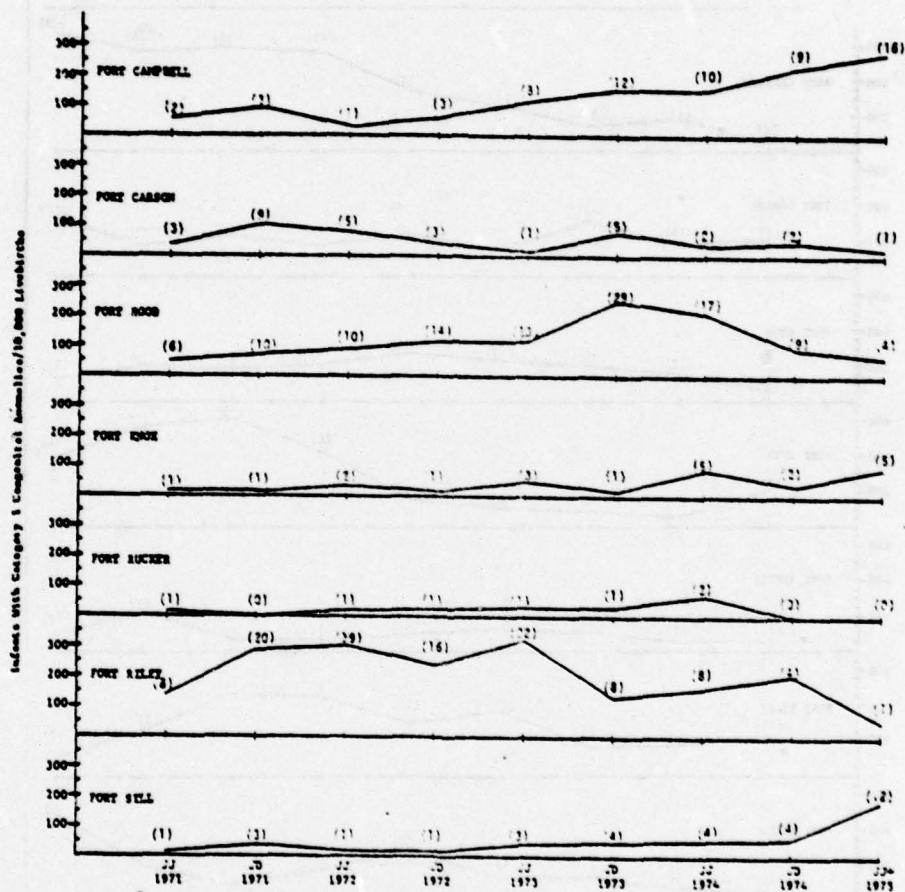


FIGURE 4 Liveborn Index with Category I Congenital Anomalies/10,000 Livebirths by Six Month Period in which The Birth Occurred For Seven Installations (1 January 1971 - 30 June 1975).

-Does not include all livebirths during the six month period.

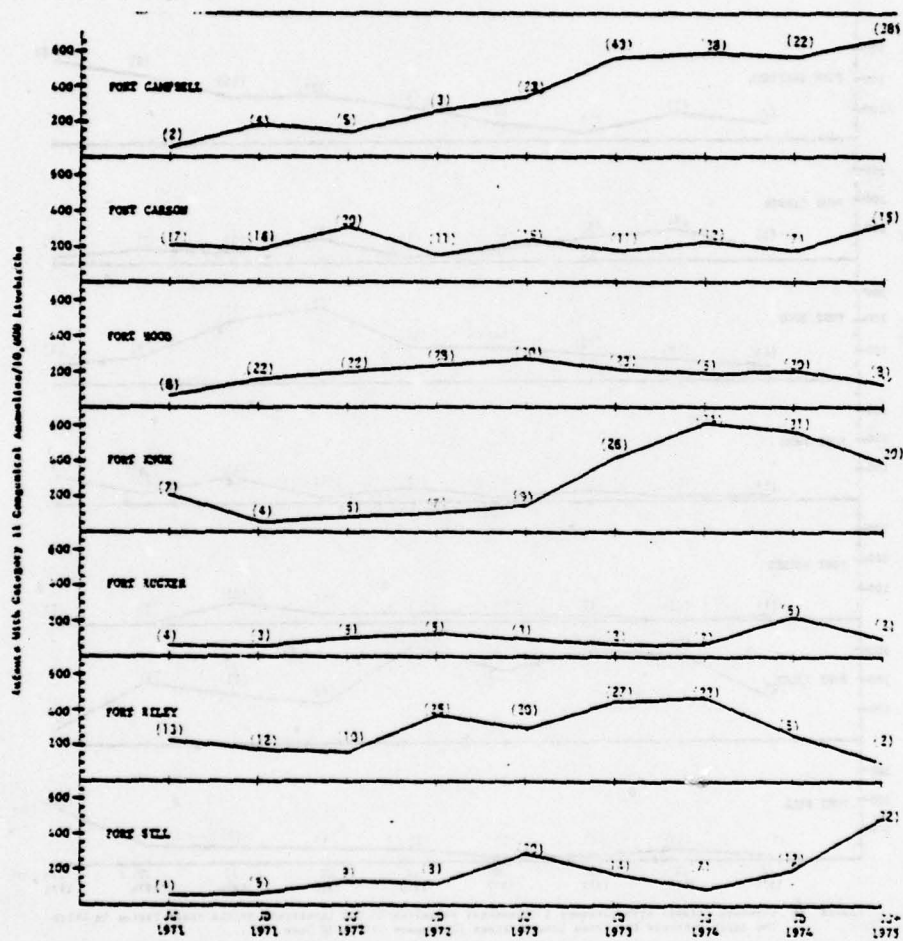


FIGURE 5 Liveborn Infants With Category II Congenital Anomalies/10,000 Livebirths by Six Month Period in Which The Birth Occurred for Seven Installations (1 January 1971 - 30 June 1975).

-Data not include all live births during the six month period.

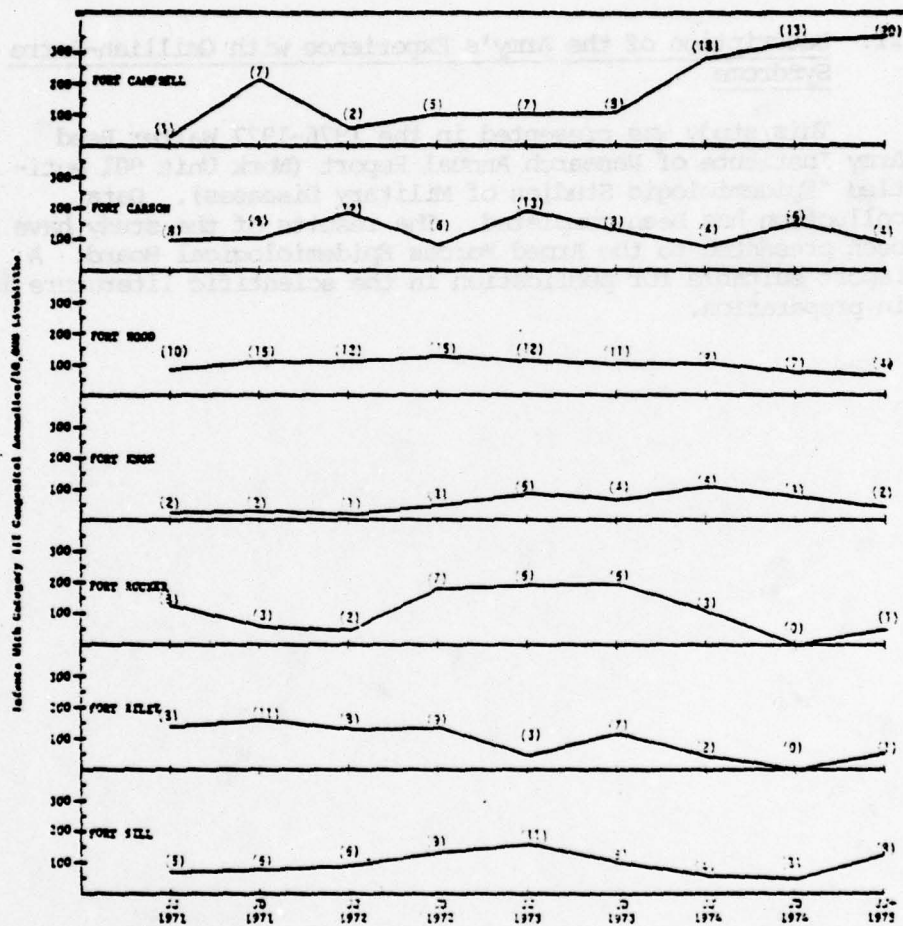


FIGURE 6 Liveborn Infants With Category III Congenital Anomalies/10,000 Livebirths by Six Month Period in Which The Birth Occurred For seven Installations (1 January 1971 - 30 June 1975).

*Does not include all live births during the six month period.

of the data generated, a decision will be made about future studies of congenital malformations. The major limiting factor in evaluating the data now available is the absence of information on infants who were delivered under CHAMPUS.

21. Description of the Army's Experience with Guillian-Barre Syndrome

This study was presented in the 1976-1977 Walter Reed Army Institute of Research Annual Report (Work Unit 001 entitled "Epidemiologic Studies of Military Diseases). Data collection has been completed. The results of the study have been presented to the Armed Forces Epidemiological Board. A report suitable for publication in the scientific literature is in preparation.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 001 Epidemiologic Studies of Military Diseases

Literature Cited.

Publications:

1. Burke, D.S., Gaydos, J.C., Hodder, R.A., Bancroft, W.H.:
Sero-immunity to Polioviruses in U.S. Army Recruits. J. Infect.
Dis., February 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
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23. TECHNICAL OBJECTIVE ^a 24. APPROACH. 25. PROGRESS (Furnish full dual paragraph identification by number. Precede text of each with Security Classification Code)							
<p>23. (U) The pathogenesis of bacterial infections of the gastrointestinal tract is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised. Diarrhea is a significant problem in military personnel operating overseas.</p> <p>24. (U) The genetic control of O-antigen specificity of enteric pathogens is being studied since such cell envelope components are of importance in disease and its prevention through vaccination. Studies of enterotoxins and other virulence determinants of shigella, E. coli and Y. enterocolitica are being pursued by a genetic approach.</p> <p>25. (U) 77 10-78-09 An Hfr donor of S. sonnei has been constructed to facilitate transfer of phase I antigen genes to other enteric genera and to prepare hybrid vaccine candidates. Shiga toxin can be released from cells through osmotic shock, spheroplasting and polymyxin B techniques. Periplasmic leaky mutants of Shigella and E. coli have been isolated which release high levels of enterotoxin into the medium. Y. enterocolitica, which is invasive (Sereny positive) and produces E. coli-like St enterotoxin, has been shown to contain two plasmids (40.7 megadaltons and 35.9 megadaltons). Strains with the 40 megadalton plasmid are Sereny positive. Loss of this plasmid results in the loss of this invasive property. No correlation of plasmid profile and the ST property has been observed in the few strains so far examined. Attempts to find a phase converting system for E. coli LT enterotoxin has failed. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.</p>							

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 002 Pathogenesis of Enteric Diseases

Investigators

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Description

The pathogenesis of bacterial infections of the gastrointestinal tract, particularly those caused by Shigella, Salmonella and Escherichia coli is being studied to establish factors and mechanisms by which disease is provoked. Through an elucidation of such elements, procedures for prevention and control of diarrheal diseases can be devised.

Progress

By employing an integrated, immunologic, cytologic and genetic approach (see previous annual reports) studies in this department are concentrating on further elucidation of: (I) virulence factors and mechanisms involved in intestinal penetration and toxin elaboration by pathogens, key mechanisms by which enteric diseases are provoked; (II) the genetic control of O antigen specificity of enteric pathogens since such cell envelope components are of importance both in disease and its prevention through vaccination; and (III) the application of genetic techniques for development of live, oral vaccines against Shigellosis.

1. Treatment of some enterotoxigenic Escherichia coli strains with the antibiotic mitomycin C resulted in lysis of the bacteria. Heat-labile enterotoxin (LT) activity of culture filtrates, determined by means of the Y-1 adrenal cell assay, increased dramatically as lysis of the culture proceeded. Further studies with E. coli strains 263 and B21-4 revealed that lysis is due to mitomycin C induction of vegetative development of a temperate bacteriophage. These findings suggest that the elevated levels of LT detected after mitomycin C treatment reflect the lytic release of cell-bound LT rather than the induction by mitomycin C of denovo toxin biosynthesis. Comparable increases in LT activity also resulted from thermal induction of a phage P1Cm lysogen of strain 263.

2. The genetic control of invasiveness and enterotoxicity of Yersinia enterocolitica is being investigated.

3. Defects in bone-marrow derived cells may occur in inbred strains of mice which are susceptible to salmonellosis.

4. The local immune response of Thiry-Vella loops to various shigella antigens has been measured.

Materials and Methods, and Results in 4 Areas of Research Named Above

1. Cellular release of heat-labile enterotoxin of Escherichia coli by bacteriophage induction

It is now well documented that some enteropathogenic Escherichia coli cause disease by colonizing the small bowel and producing enterotoxins which have been designated ST (heat stable toxin) and LT (heat labile toxin). The synthesis of these diarrhea-inducing enterotoxins by Escherichia coli can be plasmid mediated. Two fundamental classes of Ent plasmids have been recognized: one determines production of both LT and ST, whereas a second type of Ent plasmid determines only ST.

These enterotoxins can be readily detected, by use of both in vivo and in vitro assay systems, in cell free supernatant fluid following growth of the bacteria in broth media. In addition, it has recently been reported that the antibiotic mitomycin C will induce the synthesis of heat-labile toxin by E. coli, presumably by a mechanism in which LT gene(s) are derepressed and de novo toxin synthesis is initiated. This conclusion was based on the finding that supernatant fluids from such mitomycin C treated cultures yielded greater LT activity than untreated cultures.

Our studies reveal that an alternate mechanism can account for the increased LT levels following treatment of E. coli LT strains with mitomycin C. We have shown that such treatment results in lysis of the bacteria due to the induction of vegetative development of bacteriophages which can lysogenize such enterotoxigenic E. coli. The increased levels of LT in filtrates of mitomycin C induced strains thus may reflect the release of cell bound LT during lysis rather than the induction of de novo toxin synthesis.

Bacterial strains, mitomycin induction and toxin assays.
Most studies were performed with E. coli strain 263. In preliminary screening studies we also used a number of LT producing E. coli

strains associated with disease from diverse geographic areas.

For some studies with E. coli 263, we employed the thermo-inducible phage P1 Cm. This phage is a natural recombinant between Phage P1 and an R plasmid which retains its capacity to lysogenize.

For mitomycin C induction, bacteria from overnight stationary cultures were diluted 1:100 into fresh, prewarmed Penassay broth and incubated at 37°C with aeration. When growth had reached about 2×10^8 cells per ml the culture was divided. Mitomycin C (Sigma Chemical Co.) was added to one portion at a final concentration of 1 µg/ml of culture. These cells and those in the control portion were further incubated with vigorous shaking at 37°C. After overnight incubation, the cells were removed by centrifugation at 10,000 X g for 20 minutes at 4°C. The cells were discarded and the supernatant was filter sterilized (0.45 µm, Millipore Corp.) and stored at 4°C. For kinetic experiments, samples were periodically removed and sterile filtrates were prepared as described above. Enterotoxin assays were performed on such supernatant filtrates usually within 24 hours using the Y1 mouse adrenal cell system.

Treatment of E. coli 263 and other LT strains with mitomycin C.

Preliminary studies with E. coli 263 yielded findings similar to those previously described for this strain. After incubation overnight in the presence of mitomycin C, the enterotoxin activity of culture filtrates of 263 and other LT producing E. coli strains was determined. As shown in Table 1, addition of mitomycin C to cultures of 263, B21-4 and TD286C2 caused a 32-80 fold increase in LT activity of sterile supernatant fluids. The remaining strains that were tested revealed slight or no increase in LT activity as compared to untreated control cultures.

A time course of the effect of mitomycin C, on E. coli 263 is presented in Figure 1. When mitomycin C at a final concentration of 1.0 µg/ml is added to a culture of strain 263, cell growth continues to increase for 30-45 minutes at a rate typical of the control culture. At this time growth is affected and lysis of the culture is initiated, as shown by a leveling and subsequent decrease of optical density. Moreover, the LT activity of culture filtrates increase dramatically (32 fold over control) as lysis of the culture proceeded. A similar lytic event was seen with strain B21-4. In contrast, E. coli H10407, which showed no increase in LT activity after mitomycin C treatment, failed to lyse even when µg mitomycin C per ml of culture was employed.

This course of events which resulted in cell lysis was suggestive of the classic phenomenon of mitomycin C induction of bacteriophage synthesis in lysogenic bacteria. We thus considered the possibility that some LT strains harbor temperate phages which respond to mitomycin C induction. By means of an electron microscopic search, such a phage was identified. This phage, designated ϕ 263, has an isometric head with hexagonal symmetry and a short tail with no detectable contractile sheath. The phage particles appear uniform in size, with a head diameter of about 100 nm and a tail assembly of about 13 nm in width and 15 nm in length. The tail consists of 3 or more spikes and may contain a plate at the distal end.

Release of LT from *E. coli* 263 by thermoinduction of phage ϕ 263.

Our finding that *E. coli* 263 carries a temperate phage which can be lytically induced by mitomycin C, suggested that the elevated levels of LT observed in filtrates of such induced cultures might be a reflection of a release of cell-bound toxin. To determine whether lytic induction of phage synthesis can cause such an increase in LT, *E. coli* 263 was lysogenized with the thermoinducible phage ϕ 263. This phage can be specifically induced into lytic replication by elevating the incubation temperature to 42°C and inactivating the thermolabile repressor responsible for maintenance of the prophage state. The use of mitomycin C to induce lysis of 263 could thus be avoided and the levels of LT in lysates of such thermally induced ϕ 263 lysogens could be monitored. The results of such a thermal induction of *E. coli* 263 (ϕ 263) are presented in Figure 2. As cellular lysis resulting from lytic replication of phage ϕ 263 progressed, an 80 fold increase in LT activity was detected.

Our studies have shown an increase in LT activity of filtrates following mitomycin C treatment of enterotoxigenic *E. coli*. Time course studies of the effect of mitomycin C (Figures 1 and 2) provided evidence of cell lysis concomitant with the increase in LT activity. Moreover, we subsequently were able to demonstrate that this lytic event reflected the induction of bacteriophage synthesis.

On the basis of these observations, we offer an alternative mechanism for explaining the increased LT levels in supernatant culture fluids of *E. coli* strains treated with mitomycin C. We believe that mitomycin C may not specifically induce synthesis of LT; rather it induces vegetative replication of phages that may lysogenize such strains. As lysis proceeds, cellular release of pre-formed LT from the periplasmic space of the outer cell membrane

occurs, thus accounting for the increased levels of LT in filtrates.

Early in these studies we considered the possibility that temperate phages associated with toxigenic strains of E. coli may have incorporated genes for enterotoxin production into their genome. Since the structural genes for LT undoubtedly represent a small portion of the End plasmid it is likely that such LT converting phages exist. Studies in which we have lysogenized non-toxigenic strains with various phages isolated from toxigenic E. coli are currently being pursued in an effort to isolate such LT converting phages.

The use of lytic induction of bacteriophages for cellular release of toxins from bacteria may have some application to the characterization of toxins. Preliminary studies with PlCm lysogen of Shigella dysenteriae 1 strain 60R suggest that shiga toxin also can be released by induction of phage lysis. This approach may, therefore, provide an alternative to alkaline extraction or mechanical disruption of cells which are currently employed for initial enrichment and isolation of the toxin. In addition, shiga toxin isolated from phage lysates could differ from that isolated by the conventional methods and thus could provide a useful approach for delineating structurally the cytotoxic, neurotoxic and enterotoxic activities of the shiga toxin complex.

2. The genetic control of invasiveness and enterotoxigenicity of Yersinia enterocolitica is being studied in collaboration with Dr. Walter Laird (George Washington University) and Dr. John A. Wohlhieter (Dept Bacterial Immunology, Walter Reed Army Institute of Research). Investigations of two strains of Y. enterocolitica have shown that invasiveness is under the control of a plasmid. Contour length measurements of plasmid DNA isolated from strain Y7P has revealed two plasmid species; 41.93 megadaltons and 36.40 megadaltons. Curing of the 41 megadalton plasmid results in a loss of invasiveness as demonstrated in mouse and guinea pig animal models. No loss of the E. coli-like ST activity was detected in such cured clones. The function of the 36 megadalton plasmid still remains obscure. Screening studies of other strains of Yersinia enterocolitica strains by means of agarose gel electrophoresis analysis of plasmid DNA in cleared lysates reveal a correlation between the 41 megadalton plasmid and invasiveness. Strains which lacked detectable plasmids still expressed ST activity, suggesting that, unlike in E. coli, ST genes of Yersinia are chromosomal.

Table 1. Enterotoxin (LT) activity after treatment with mitomycin C^a

E.coli strain	LT activity (U/ .05 ml) ^b		Increase in activity (+/-)
	-Mitomycin C	+M tomycin C	
263	8	640	80
H10407	4	4	
B21-4	10	320	32
TD427C1	10	10	
334a	10	20	2
TD504C1	10	20	2
B2C	2	10	5
TD286C2	2	160	80

^a Treated with 1 µg of mitomycin C per ml.

^b LT activity = reciprocal of highest dilution of filtrate causing Y-1 cell rounding.

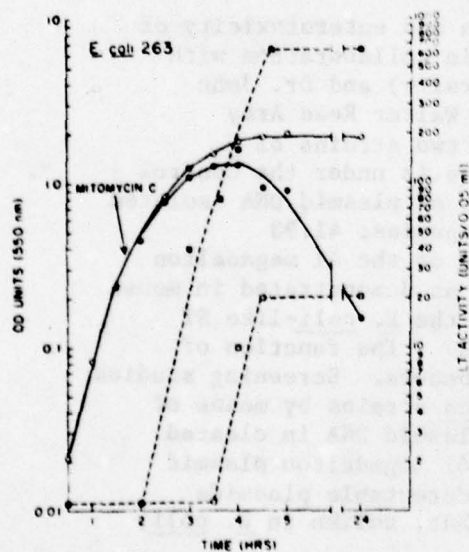


FIG. 1. Induction of lysis of *E. coli* 263 by mitomycin C. Samples were removed and assayed for enterotoxin, as described. Symbols: Control (no mitomycin C); ○, OD₅₅₀; and □, LT activity; mitomycin C added: ●, OD₅₅₀; and ■, LT activity.

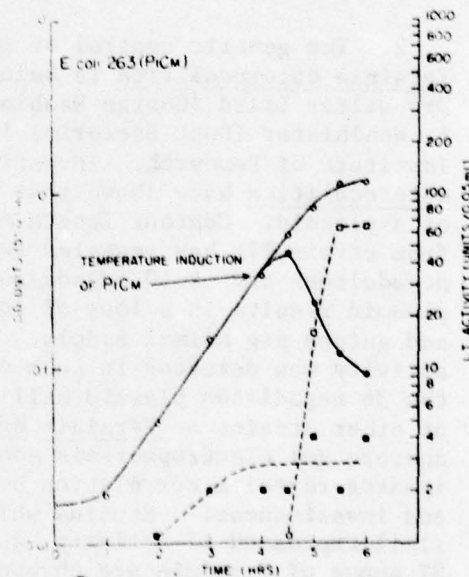


FIG. 2. Thermal induction of *E. coli* 263 (PICM). A PICM lysogen of *E. coli* 263 was grown to a concentration of about 3×10^8 cells/ml at 32°C with vigorous aeration. At this time, the culture was split, and one portion was thermally induced by incubation at 42°C for 14 min with vigorous aeration. This culture and the control portion were then incubated further at 32°C. Symbols: Control ○, OD₅₅₀; ■, LT activity. Thermal induction: ●, OD₅₅₀; □, LT activity.

3. As mentioned in the last annual report, we have described two genes which influence the response of inbred mice to Salmonella typhimurium infection, an X-linked gene expressed by the B-lymphocyte defective CBA/N strain of mice) and a lipopolysaccharide (LPS) defective response gene expressed by the C3H/HeJ strain of mice. During the last year we have tried to determine if expression of the immune defects of these two strains of mice is actually linked to increased susceptibility of S. typhimurium. Preliminary results of such linkage studies indicate that with CBA/N mice, expression of the B lymphocyte defect (as assessed by decreased serum IgM levels) correlates with an increase in susceptibility to murine typhoid among backcrossed mice bred specifically for this study. The breeding of backcross mice for linkage studies with the C3H/HeJ model is as yet not complete. We have also shown that with both CBA/N and C3H/HeJ mice resistance to S. typhimurium can be transferred to lethally irradiated immune-defective mice with bone marrow from the appropriate syngeneic immunologically normal S. typhimurium resistant control. These studies suggest that with both models the genes governing the increased susceptibility to S. typhimurium are expressed by bone-marrow derived cells.

4. The Thiry-Vella loop model has continued to be used to study the local immune response of the intestine to various shigella antigens. The enzyme-linked immunosorbent assay has been employed to assess the immune response.

Our studies have demonstrated that the presence of a Peyer's patch enhanced the early local immune response, but its presence was not necessary to achieve a strong local response. Furthermore, we pointed out the similarity of the epithelium overlying the Peyer's patches with that overlying isolated lymphoid follicles which occur in large numbers throughout the intestine, and suggested that these smaller structures may also contain precursors for IgA-secreting plasma cells.

Another factor which has a more important effect on the immune response is the time of antigen administration in relation to the time of the construction of the loop. When the Thiry-Vella loops were given 10^8 live Shigella X16 on days 6 and 13 after surgical creation of the loops, little local immunity was detected in secretions. However, when the animals were immunized on days 1, 8 and 15 after surgery, strong local responses were seen by day 11 (i.e. before the third dose). The dosage schedule of days 1, 8 and 15 was adopted for all subsequent studies.

One factor which may have been important with regard to

the local immune response is the degree of colonization of these loops by bacteria. Whereas the normal intact rabbit ileum contains relatively small numbers of anaerobic organisms, we have determined that our chronically isolated ileal loops are rapidly colonized with bacteria. The loops remain relatively sterile for the first two days after isolation, but by the 6th day after surgery, over 90% are colonized, with an average of 10^8 Pseudomonas aeruginosa.

Since a considerably stronger secretory immune response was found in loops immunized with Shigella X16 before they were colonized by P. aeruginosa than those that were immunized after they were colonized with P. aeruginosa, we asked whether the pseudomonas was somehow inhibiting the local immune response.

For this study, 5 rabbits with chronically isolated ileal loops were colonized on the day of surgery with 10^8 live P. aeruginosa. The continued presence of this organism was documented by cultures of the daily loop secretions. Therefore, on day 1 after surgery, these animals had the flora usually not present until day 6. All animals were immunized on day 1, 8 and 15 after surgery with 10^8 live Shigella X16. A strong local immune response was found in these animals to the Shigella X16, as was found in animals that were not artificially primed with Shigella X16.

Another factor which could account for the enhanced local immune response to Shigella X16 administered on days 1, 8 and 15 v.s. the response to Shigella X16 given on days 6 and 13 would be the inflammation and repair process. On the first day after surgery there is considerable inflammation, but by the sixth day, virtually all the acute inflammation due to surgery has subsided, although some fibroblastic repair is still occurring at the periphery of the loops.

To determine whether this acute inflammation is responsible for the enhanced immunity, 6 rabbits had ileal loops created. After 5 days, the loops were reoperated upon - a small cuff of intestine at each end of the loop was excised. This, then, would recapitulate the conditions of acute inflammation in loops that had aged 5 days, and which had the flora, normally found at 5 days. Then, on day 6, after the original creation of the loops (but day 1 after the second operation), the loops were stimulated with 10^8 live Shigella X16. If the inflammation, due to the surgery is responsible for the enhanced immunity in rabbits on days 1, 8 and 15 after surgery, this group of rabbits should display a strong response. If, however, the poor response in

rabbits given antigen on days 6 and 13 relates to atrophy of these these loops, a poor response will be achieved.

These experiments are currently in progress.

Since our early work demonstrated an atrophy of villi with time in these isolated loops, we were concerned as to whether or not the follicle associated epithelium (FAE) which overlies Peyer's patches and presumably function in processing gut antigens might atrophy as well. Therefore, a chronically isolated ileal loop was perfused with 50:50 mixture of ferritin and India ink. On electron microscope examination, as much ferritin was taken up by the FAE of this loop as by the FAE of intact small bowel in the same animal.

The chronically isolated ileal loop model was employed to determine whether a single oral or subcutaneous dose of antigen would "prime" the bowel and thereby enhance the secretory immune response to subsequent local administration of that same antigen, six rabbits were given 10^{10} live Shigella X16 intravenously and six other rabbits received 10^{10} live Shigella X16 orally. After 30 days, a chronically isolated ileal loop was created in each animal. The loops were stimulated with 10^8 live Shigella X16 on days 1, 8 and 15 after surgery. Good local immune responses were present in both groups. No enhancement or suppression of the time course or strength of local response was observed when compared to unprimed animals that were stimulated on days 1, 8 and 15.

Although no evidence of an anamnestic response was found in these animals, we were not certain that an adequate oral or subcutaneous priming had been achieved.

To further explore the question of anamnestic response, we immunized 4 animals by the intraloop route on days 1, 8 and 15 after surgery and followed the kinetics of their local immune response. When the level of local antibody to Shigella X16 returned to "baseline", the animals were restimulated with a single dose of 10^8 live Shigella X16 intraloop. Their responses were again recorded until baseline was achieved. Three of the four animals achieved strong IgA anti-Shigella X16 levels within 4-5 days after this remote stimulation (as long as 200 days after the initial local dose), whereas to achieve this level of response initially required at least 10 days and two doses of antigen. The fourth animal was inadvertently restimulated before its IgA anti-X16 levels had returned to baseline. Interestingly, though, it also had an apparent rise in IgA anti-X16 activity following this intraloop

dose, the rise was smaller than that found in the other three animals.

All the above immunologic studies were performed using *Shigella* X16 which is a locally invasive organism. It was of interest to learn whether the invasion of the tissue is necessary, or whether a local immune response could be obtained to a non-invasive strain of *Shigella*. Therefore, 10^8 live strain 2457-0 of *Shigella flexneri* (a noninvasive bacteria) were used to immunize isolated ileal loops in 6 rabbits. All rabbits produced a strong local immune response to the 2457-0 LPS. The kinetics and strength of the local immune response were similar to those found for the invasive *Shigella* X16.

Since a noninvasive strain could produce a strong local immune response, we asked whether the LPS alone could elicit local antibodies. LPS from *Shigella* X16 (a Westphal preparation) at a concentration of 100 $\mu\text{g/ml}$ was used to immunize 6 rabbits on days 1, 8 and 15 after surgery. No IgA anti-X16 activity was detected in any secretion. Studies are currently underway to determine whether killed bacteria are able to elicit good local immune responses.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 002 Pathogenesis of Enteric Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				AGENCY ACCESSION#	DATE OF SUMMARY	REPORT CONTROL SYMBOL DD-DR&E(AK)36	
				DA 0 6446	78 10 01		
1. DATE PREV SUMMARY	2. KIND OF SUMMARY	3. SUMMARY ACT	4. WORK SECURITY	5. NEUTRAL JO	6. USER NUMBER	7. SPECIFIC DATA CONTRACTOR ACCESS	8. LEVEL OF USE
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ADDRESS* Washington, DC 20012				ADDRESS* Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursue DDAR II U.S. Address Institution)			
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(U) Immune Responses; (U) Shigellatoxin; (U) Intestine; (U) Electron Microscopy (EM)							
23. (U) To define histopathologic manifestations of injuries and diseases which have current or potential problems in military personnel. The current effort is directed toward studies of enteric diseases and immunologic responses with infections. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in enteric infectious diseases of military personnel.							
24. (U) Various morphologic techniques including histology, histo- and cytochemistry, autoradiography, immunofluorescent microscopy, transmission and scanning electron microscopy are employed. Various immunologic techniques have also been utilized.							
25. (U) 77 10-78 09 Light and EM studies on the response of the guts to shigella toxin have been in progress. Transmission and scanning EM studies have demonstrated that cryptosporidia, a species of intestinal coccidia attach and fuse the microvilli of the gut epithelial cells. For the first time, experimental meningococcus and gonococcus infections have been established in the lung of guinea pigs and mice. Collaborative EM studies in rickettsia - host cells in vitro and vivo demonstrated cellular changes of infected cells. EM studies by freeze-etching and thin-sectioning techniques on Trypanosoma have defined the details of the pocket of flagella of this motile parasite. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.							

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Project: 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 003 Histopathologic Manifestations of Military Diseases and Injuries

Investigators:

Principal: Akio Takeuchi, M.D.

Associates: Edwin Ewing, MAJ, MC, Tatsuo Hase, M.D., SSG Garnett Henley, MS, E4 Lindsey Inman, BS, Paul Hildebrandt, COL, VC

Description:

To define histopathologic manifestations of injuries experimentally produced and diseases which present current or potential problems in military personnel. The current effort is directed toward studies of diseases of the digestive tract and immune responses due to infections and injuries. These studies provide a basis for a comprehension of pathogenesis, scientific treatment, and determination of prognosis in diseases and injuries in military personnel.

Approach to the Problem:

A multi-disciplinary approach including conventional histology, histo- and cytochemistry, autoradiography, radio-tracer methods, various immunological techniques, immunofluorescent microscopy, transmission and scanning electron microscopy is employed.

Progress:

This work unit consists of studies of histologic manifestations of acute diarrheal diseases of infectious origin and collaborative studies of experimental gonococcal, rickettsial and trypanosomal infections with other departments of the WRAIR.

I. Studies of Host-Pathogenic Microbe Relationship In The Digestive Tract.

A. Studies on the Effects of Enterotoxin Derived from Shigella dysenteriae on the Small Intestine of Rabbits.

Background:

The virulence of shigellae is thought to depend upon their ability to penetrate intestinal epithelial cells and to persist

within the mucosa of the intestine. Invasion of the colon results in the characteristic colitis and symptoms of dysentery that are the hallmarks of shigellosis. In most patients, however, a period of watery diarrhea precedes the onset of dysentery and in some way may be the sole manifestation of disease.

Several lines of evidence suggest that the diarrheal phase may relate to involvement of the small bowel. Studies of gastrointestinal intubation in man have demonstrated that two other organisms which cause watery diarrhea, Vibrio cholerae and Escherichia coli, are present in high concentration in the fluid of the small bowel, where there is net secretion of water and electrolytes into the lumen. Similarly, organisms have been isolated from ileal fluids during the diarrheal phase of induced shigellosis in man. When dysentery develops, organisms may be recovered from the colon. An identical sequence has been seen in early experimental shigellosis in the guinea pig, in which evidence of epithelial penetration into the ileum was seen. Similarly, inoculation of virulent shigellae into isolated segments of adult rabbits results in accumulation of fluid and dilatation of the loop. One species, Shigella dysenteriae, elaborates a protein enterotoxin *in vitro* which also produces net secretion into the rabbit ileum. In collaboration with Department of Bacterial Diseases, WRAIR, the present study was undertaken to determine the effects of shigella enterotoxin on the morphology of rabbit ileum.

Materials and Methods:

One-hundred-five ligated ileal segments in 23 white New Zealand rabbits were constructed. In each animal separate segments were inoculated with buffer, active shigella enterotoxin (5- and 50-ug doses), and shigella enterotoxin inactivated by heating at 90C for 30 min. Five animals were killed at 1, 2, 4 and 6 hr and three more 12 hr after toxin was placed in the intestinal lumen. In eight animals (two each at 1, 4, 6, and 12 hr), both cholera and shigella toxins were placed in different loops in individual animals for comparative study. Representative sections were cut, stained and examined with the light microscope.

One hour after exposure to 50 ug of shigella enterotoxin, the ileal mucosa showed minimal focal changes, characterized by discharge of mucus from goblet cells and degeneration of focal epithelial cells at the apex and mid-villous portion of the villi. These changes were associated with increased cellularity of the lamina propria which intensified as time progressed. The morphologic alterations remained qualitatively similar at 2, 4, and 6 hr; for this reason we have chosen to illustrate only the fully developed lesion.

At 6 hr after exposure, there was definite shortening of the villi with a decrease villus-to-crypt ratio. Individual epithelial cells, or small clumps of altered cells were in the process of extrusion into the lumen, from the side as well as the tip of the villus. There was marked disarray of the epithelial cell layer, characterized by many necrotic cells, focal erosions, and large numbers of intact or degenerating lymphocytes caught in transmigration. The brush border was sparse and irregular in some areas. The epithelium, particularly near the villus tip, became cuboidal and often appeared as a cap of altered cells. There was considerable nuclear debris scattered throughout the epithelial layer and lamina propria. In the same animals, exposure to cholera enterotoxin failed to alter ileal morphology.

Ileal sections from animals killed 12 hr after instillation of toxin had artifactual changes due to distention of the fluid-filled intestinal loop and appeared similar to loops treated with cholera enterotoxin.

Recommendation:

Further ultrastructural studies of this experimental model will assist in the clarification of the nature of cytotoxic effects of intestinal epithelial cells by shigella enterotoxin.

B. Studies on Cryptosporidia Infection in the Small Intestine of Rabbits.

Background:

The genus, *Cryptosporidium*, was introduced by Tyzzer in 1907 with the description of the type species *C. muris* in the common mouse. In his original article and in subsequent publications (1910, 1912) Tyzzer mentioned a *Cryptosporidium* similar to *C. muris* in the rabbit small intestine, however, he did not elaborate. Since that time several species of cryptosporidia have been described in various mammals including guinea pig (Jervis et al., 1966), lamb (Barker, 1974), calf (Barker, 1974, Meuten, 1974) and rhesus monkey (Kovatch, 1972, Cockrell, 1974). Recently, cryptosporidiosis associated with acute diarrhea was reported in human patients (Meisel, 1976, Nimi, 1976).

Since cryptosporidial organisms have not as yet been cultured, we have studied spontaneous infections in the small intestine of adult female rabbits to provide additional information on host-parasite interrelationships and mechanisms of tissue damage.

Observations:

Juvenile female rabbits (*Oryctolagus cuniculus*, Walter Reed strain) had no clinical signs of enteric disease at time of reception, and were placed on experiment. The animals were killed by injection of a lethal dose of pentobarbital, and tissue from the loop used in *Shigella* enterotoxin studies and a control area proximal to the loop were taken and processed for light (LM), transmission (TEM), and scanning (SEM) electron microscopy.

A low magnification, LM examination on histological sections of the ileum showed a shortening and blunting of villi with a moderate decrease in the crypt to villous ratio. A slight edema in the lamina propria and dilation of the lacteals were also apparent. Numerous lymphocytes in transmigration were found in the epithelium. At higher magnification, Giemsa stained sections had multifocal aggregates of strongly basophilic, round-to-ovoid bodies embedded within the brush border at the apical portions of villi (Fig. 1), and strongly resembled a cryptosporidia previously observed in guinea pigs at this laboratory (Jervis et al., 1966). These same structures could be seen, less distinctly stained, in H&E sections.

TEM observations showed the characteristic ultrastructure of various stages of a species of *Cryptosporidium*. Microvilli were completely absent wherever the organisms were located along the epithelial surface. The organisms were closely attached to the cell surface displacing or replacing the microvilli. Adjacent microvilli showed at times some apical blebs or were slender, irregularly shaped, and closely associated with the parasite. There is an electron translucent attachment zone (Fig. 2) between parasite and host cell membranes. The intracellular structures of the parasites such as inclusions, nuclei, and the intraparasitic membranous folds were similar to those previously described.

At magnification greater than 20,000X, it appeared that the organisms were surrounded by a double host cell membrane. Just below the host membrane at the site of parasite attachment, a dense band is noted that occasionally demonstrated a regular serrated pattern (Fig. 2). Several stages of the life cycle of cryptosporidia were recognized from the implanting merozoite, to shizonts and oocytes; these were comparable to those illustrated by other investigators.

SEM observations offered a new perspective on the relationship of the parasite to the gut epithelium. At low magnification, the organisms could be seen around structures embedded within the brush border. More noticeable under SEM was the presence of elongated

microvilli in close proximity to the organism. At higher magnification, membrane derivation suggested by TEM could be better visualized by SEM. Unique surface structures approximately the width of adjacent microvilli could be seen when a favorable viewing angle was obtained (Fig. 4). These structures appeared to be continuous with the outer membrane of the parasite. At similar magnifications, mature schizonts were identifiable. Their 8 merozoites could be seen in a neatly arranged octet presumably held in that arrangement by a central residual mass.

Comment:

The parasite structurally resembles *C. wairi* from the guinea pig (Jervis et al.) and other cryptosporidia described in other species. However, if we assume species specificity (Nimi, 1972, Kovatch, 1972, and Vetterling, 1971), it seems likely that this organism belongs to the same species as the organism first reported by Tyzzer in the small intestine of the rabbit which he assumed to be *C. parvum*.

From our TEM observations, it appeared, in all stages, with the exception of the merozoite, that the organism was intracellular, surrounded by a double membrane of host cell origin. This agrees with the data presented by Vetterling et al. (1970) and later by Cockrell (1974) for other cryptosporidia. However, SEM micrographs strongly suggest the possibility that we were viewing tangential sections and that the organisms were actually outside the cells.

The process involved in the attachment of the cryptosporidia or their penetration of the epithelial cells is perhaps the most intriguing problem which the organism presents. It never occurs in areas below the level of the base of adjacent microvilli and yet it appears to be surrounded by a double host membrane. It suggests that the envacuolization may occur by a process in which the host microvilli envelop the organism (Fig. 3) and fuse with adjacent microvilli (Fig. 4).

The rabbit is an economical and popular animal for study of enteric pathogens. In this laboratory, we have seen several cases of cryptosporidial infection in the rabbit small intestine which limits its value as a research animal.

Conclusion and Recommendation:

LM, TEM and SEM observations have provided additional information on host-cryptosporidia relationship in the small intestine. For the first time, SEM observations have demonstrated a unique interaction with the microvilli of the intestinal epithelial cell.

II. Studies on Experimental *Neisseria gonococcus* Infection

Background:

Numerous investigators have tried to produce experimental *Neisseria* infection in various animals by different modes of infection with little success. Recently, we were able to produce infections in adult mice and guinea pigs by intranasal inoculation of freshly isolated *Neisseria gonococci* from human patients. Preliminary observations have shown that mice inoculated with approximately 10^7 - 10^8 gonococci developed acute pneumonia with mild leukocytic infiltration.

In collaboration with Department of Bacterial Diseases, we are continuing to develop this experimental model to better define the mechanisms of acute purulent inflammation, host cell-bacterial relationship and immunopathology of this infection.

Preliminary Results:

After intratracheal inoculation and sequential examination of the lung of mice with *N. gonococcus* infections, a progressive increase of bacteria with a minimum of parenchymal degeneration and inflammation was found (Fig. 5). In contrast, guinea pigs similarly challenged developed an acute bronchopneumonia characterized by progressive acute inflammation with parenchymal degeneration at 24 hours post-infection. However, the bacteria are usually absent (Fig. 6).

Post-infection	2 hrs	4 hrs	6 hrs	24 hrs
Mice	Acute \pm inflam- mation	Acute \pm inflam- mation	Acute \pm inflam- mation	Acute - inflam- mation
	Bacteria +	Bacteria +	Bacteria $^{+++}$	Bacteria ++
Guinea Pig	Acute \pm inflam- mation	Acute \pm inflam- mation	Acute \pm inflam- mation	Acute $^{+++}$ inflam- mation
	Bacteria +	Bacteria +	Bacteria \pm	Bacteria \pm

We will expand the study on this model to determine the mechanisms causing the difference of tissue reactions and the role and fate of the bacteria in infected mice and guinea pigs.

III. Studies on Rickettsial Infection

A. Studies on Stylostome Formation by Leptotrombidium Mites (ACARI: Trombiculidae).

Background:

There are at least seven species of trombiculid mites, all in the subgenus Leptotrombidium, that vector Rickettsia tsutsugamushi, the etiologic agent of scrub typhus. Considerable information exists on the taxonomy, ecology, and bionomics of these mites, however, a little is known about the mechanism of the mite-host interrelationship that results in transmission of R. tsutsugamushi to the vertebrate host. It was suggested that the cellular environment at the site of larval feeding may be a significant factor in transmission of rickettsiae both from and to the mite.

For these reasons, in collaboration with Department of Hazardous Microorganisms, WRAIR, we studied histologic changes in mouse skin parasitized by the larvae of both vector and nonvector species of Leptotrombidium in order to characterize the local host tissue reactions at sites of feeding and also to define the stylostome or feeding tube.

Materials and Methods:

Unengorged larvae of the various Leptotrombidium species were applied to the ears of white laboratory mice. Mice were then held at room temperature and killed at 24, 48 and 74 hr intervals. Ears were excised, fixed and examined.

The mites were reared in a dual program incubator programmed to provide alternating periods of 12 hrs of illumination at 30C followed by 12 hrs in darkness at 25C with relative humidity at or near saturation throughout (e.g., conditions of temperature, humidity, and photo-periods similar to those of a typical Malaysian habitat).

Results:

Classification of stylostome formation. Typical pictures of sections of mouse skin with larvae attached are shown in Fig. 7. At 24 hrs after attachment, stylostomes were well-formed and showed characteristics of each species of mite involved. Based on distinctive differences in histological morphology, stylostomes were classified into three types. The first type, the epidermal stylostome, was characteristic of L. intermedium; the second, the mesenchymal

stylostome, was characteristic of L. fletcheri; and the third type, was the mixed stylostome characteristic of L. arenicola and L. deliense.

The epidermal stylostome. The stylostome formed by individual L. intermedium was a cone-shaped structure which consisted of a central canal, an inner wall of chromophobic materials, and an outer wall of incorporated epidermal tissue. The material which constituted the inner wall was a mass of uniform thickness, distinctly chromophobic to both H&E and Giemsa stains, appearing as a homogeneous, non-staining material around the central canal. In this respect, it differed from eosinophilic hyaline material seen in the mammalian tissue. It was probably secreted by the mite. The outer wall made of host epidermal elements molded the whole stylostome into a cone-shaped structure with its tip pointed at the epidermal-dermal junction. The outer wall consisted of layers of keratin and parakeratotic cells. Evidence of fresh incorporation of epidermal cells was seen in the outermost layer. Thus, the hyperplastic epidermis, which showed spongiosis in the prickle-cell layer, contained the whole structure of the stylostome. The stylostome penetrated the entire depth of the hyperplastic epidermis, with its tip opening to tissue spaces developed at the epidermal-dermal junction. The stylostome did not extend beyond the epidermal-dermal junction.

The mesenchymal stylostome. The initial stage of the formation of stylostome in L. fletcheri appeared to be similar to that of L. intermedium. At a certain stage of its penetration into the epidermis, however, it appeared to evoke a marked chemotactic effect on leukocytes and became completely surrounded by a dense aggregate of inflammatory cells. As a consequence, an extension of stylostome penetrated the dermis together with the sheath of inflammatory cells. The mesenchymal stylostome therefore was composed of a tube of chromophobic material of mite origin. Instead of a thick coat of epidermal tissue, a thin rim of eosinophilic hyaline material of probable inflammatory tissue origin was recognized over the outer surface of the tube. The inner surface was coated by a thin rim of eosinophilic material, probably derived from a combination of host tissue fluid and salivary secretions of the mite.

The mixed stylostome. The stylostome of L. arenicola and L. deliense were very similar in histological appearance. This type of stylostome showed the characteristics of an epidermal stylostome in the epidermal portion and those of a mesenchymal stylostome in the dermal portion. The mixed stylostome incorporated epidermal elements less conspicuously into its epidermal portion than the epidermal stylostome, and showed a sheath of inflammatory cells

surrounding epidermal elements. The dermal portion of the mixed stylostome tended to extend in parallel to the overlying epidermis.

Evaluation of the host lesion and the fate of the stylostome. All stylostomes were surrounded by a considerable inflammatory reaction in the underlying dermis at 24 hrs and 48 hrs, which consisted of vascular dilatation, extravascular exudation, and inflammatory cell infiltration. Dermal inflammation was most pronounced in the mesenchymal stylostome, intermediate in the mixed stylostome and least pronounced in the epidermal stylostome. Degranulated mast cells were frequently seen in the inflammatory area of the dermis.

After the mite left the host at 72 hrs, the epidermal stylostome showed degeneration and keratinization of the outer wall. The stylostome was walled off by a sheet of epidermal cells and thereafter expelled from the skin. With the sloughing of the stylostome, dermal inflammation subsided rapidly. Upon detachment of the chigger, the mesenchymal stylostome remained embedded in host dermal tissue. This stylostome, surrounded by granulomatous tissue, was probably expelled from the host when overlying tissue ulcerated.

Comparison of the lesion resulting from the feeding of either normal or infected mites. Normal and infected larvae of L. fletcheri both produced typical mesenchymal stylostomes. The presence of R. tsutsugamushi in the mite did not appear to alter the histologic picture at the site of feeding during the study. Small particles resembling rickettsiae were apparent in large mononuclear cells adjacent to stylostomes of infected mites in Giemsa-stained sections; however, in this study the particles could not be specifically identified as R. tsutsugamushi.

Comment:

There is a general agreement on the process of initial penetration of the skin by the trombiculid mite larva. They attach themselves to the host skin by piercing the horny layer with the cheliceral blades, apply their buccal aperture to the surface of the host skin, and seal the juncture with a hyaline mass which in time solidifies into a solid gel. Observations on living mites have shown that they then inject a complex salivary secretion, which penetrates the epidermal layer and rapidly gels to form a hyaline tube with a central canal or stylostome (Jourdain, 1899, Jones, 1950, Willmann, 1955, Schumacher and Hoeppli, 1963, and Cross, 1964). During feeding, the mite alternates phases of suction of host tissue juice and ejection of its salivary secretions through the stylostome. The digestive fluids probably contain

lytic enzymes and anti-coagulants that cause disruption of host epidermal cells (Jones, 1950).

The chemical property of the chromophobic gel of mite origin is not clearly defined. Histochemically, proteins, lipids, neutral polysaccharides, keratin, keratohyaline, and nucleic acids have not been demonstrated (Aoki, 1957 and Voigt, 1970). These negative tinctorial properties suggest that the stylostome per se does not originate from host tissue. Positive tinctorial properties show a fibrillar nature as revealed by elastic and reticulum fiber stains (Aoki, 1957, and Voight, 1970). and an acid polysaccharide component (Schumacher and Hoeppli, 1963, and Voight, 1970).

Some confusion has arisen concerning the morphogenesis of the stylostome due, perhaps, to the fact that stylostomes were studied without particular regard to the different species involved (Gudden, 1871, Andrea, 1927, Feng and Hoeppli, 1933, Jones, 1950, Aoki, 1957, Schumacher and Hoeppli, 1963, and Voight, 1970). While Voight (1970) stated that there are no differences in the development of the stylostomes within the various kinds and species of trombiculids, stylostomes in our studies showed distinctive characteristics of an individual species. For example, the stylostome of L. fletcheri can be clearly distinguished from those formed by L. intermedium, L. arenicola, and L. deliense. However, the stylostomes of L. arenicola and L. deliense could not be differentiated from each other. This proves that in this type of investigation we must deal with as divergent species of trombiculid mites as possible.

It has been generally accepted that the stylostome is formed exclusively of chigger secretion. In contrast, the present study has demonstrated that stylostome formation appears to result from an interaction of both chigger secretion and host tissue. Further, the type of stylostome formed may depend on the composition of salivary secretions injected by a particular species of chigger. Jones (1950) in studies of feeding tubes confined to the epidermis, concluded that the eosinophilic hyaline mass of the stylostome is not formed of solidified mite secretion, but is principally composed of superimposed layers of keratinized host tissue deposited along the track of the irritant mite secretion. From this it is evident that Jones has only described the epidermal tissue elements of the epidermal type of stylostome. We found that this type incorporates an impressive amount of host epidermal tissue elements around the basic tubular structure of chromophobic material secreted by the chigger. In contrast, the mesenchymal stylostome of L. fletcheri incorporated a minimal amount of eosinophilic hyaline material of probable host origin, suggesting a varied tissue response resulting from a species specific salivary secretion.

Host skin reacts to a penetrating stylostome in two ways: epidermal hyperplasia and dermal inflammation. Whether or not the chigger secretes an active substance which incites proliferation of epidermal cells is unknown at present, although Jones (1950) has reported that epidermal hyperplasia resulted from an irritant secretion of the larvae. Inflammation seems to be caused by some specific substance produced by the feeding larval mite since, while intradermal injection of homogenized L. fletcheri larvae does not produce appreciable inflammation at the site of inoculation, dermal inflammation at the feeding site extends considerably from the stylostome. Indeed, chiggers which form mesenchymal or mixed stylostomes apparently secrete a potent chemotactic substance because these stylostomes are rapidly surrounded by dense aggregates of inflammatory cells even while still in the epidermal layer. Inflammation at the feeding site of mite larvae unquestionably enhances the efficiency of the feeding process by breaking down tough, collagenous dermal tissue. The abundant tissue fluids containing numerous wandering cells would seem to be an entirely suitable milieu for R. tsutsugamushi.

It is interesting that the only species in this study that produced an epidermal stylostome, viz. L. intermedium, has never been shown to transmit R. tsutsugamushi. L. fletcheri, L. arenicola, and L. deliense, all proven vectors, formed mesenchymal or mixed types of stylostomes. Certainly, the induction of inflammatory changes in the tissue adjacent to the stylostome seem favorable both for uptake of rickettsiae from infected hosts and for rickettsial transmission to the host. Therefore, the mesenchymal and mixed stylostomes which cause severe inflammation, penetrate deeper into the host tissue and leave residual granulomatous foci, in comparison with the epidermal stylostome, which appear to preclude a favorable condition for rickettsial transmission in the host dermal tissue.

Conclusion and Recommendation:

The modes of stylostome formation by larvae (chiggers) of Leptotrombidium intermedium, L. fletcheri, L. arenicola, and L. deliense in parasitized mouse skin were studied histologically in relation to their capacity to transmit Rickettsia tsutsugamushi. Based on dermal inflammations, three types of stylostome formations were histologically defined among the different species: the epidermal stylostome formed by the larva of L. intermedium; the mesenchymal stylostome formed by the larva of L. fletcheri; and, the mixed stylostome formed by the larva of both L. arenicola and L. deliense.

Additional studies with vector and non-vector species are

indicated to confirm the possible correlation between type of stylostome and vector efficiency.

B. Electron Microscope Study of Mouse Capillary Endothelium Infected with Rickettsia tsutsugamushi.

Rickettsiae enter the bloodstream and infect capillary endothelial cells throughout the body. Yet little is known about the morphologic character of the resultant vascular lesions.

For this reason, we have been studying the capillary endothelium of BALB/c mouse cerebellum to ascertain the suitability for study of these lesions by TEM. Mice were sacrificed 6 days after intravenous inoculation of 1.2×10^6 50% mouse lethal doses of egg-grown R. tsutsugamushi, strain Karp.

Results:

Rickettsiae were identified free within endothelial cytoplasm. When infected and control endothelial cells were compared, infected endothelial cells were swollen and contained increased numbers of mitochondria and lysosomes. Occasional giant mitochondria were found in infected cells. There were focal perivascular hemorrhages and intravascular fibrin thrombi. However, the junctional complex and basal lamina of endothelium and pericytes remained unchanged.

These results suggest that capillary endothelial cells respond to rickettsial infection with hypertrophy rather than degeneration and their cytoplasmic changes represent a morphologic expression of altered cellular metabolism of infected cells.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 003 Histopathology Manifestations of Military Diseases
and Injuries

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Work Unit 003 Histopathologic Manifestations of Military Disease
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Fig. 1. Cryptosporidiosis of the small intestine of a rabbit. Round structures represent cryptosporidia at the brush border of epithelial cells. X600.



Fig. 2. Electron micrograph showing a sporozoite at the brush border of an epithelial cells. Adjacent to the parasite, the microvilli (MV) are elongated. Mitochondria are swollen and show altered cristae. X14,000.



Fig. 3. Low magnification scanning electron micrograph of proximal ileum showing cryptosporidia embedded in the brush border of several epithelial cells. This view also shows microvillar changes in many of the parasitized cells. X4,000.



Fig. 4. Mature schizone with eight distinct merozoites. Tyzzer (1912) saw these in stained cecal scrapings and thought they were held together by a central mass of residual material. X34,000.

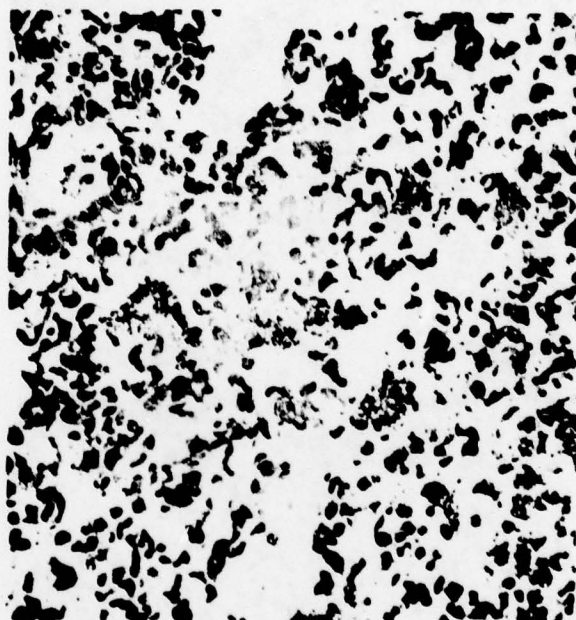


Fig. 5. Lung, mouse infected intratracheally with *N. gonococcus*, 12 hours post-challenge. Aggregates of bacteria are mainly in the alveoli and some appear within macrophages. Note that there are few inflammatory cells. X400.

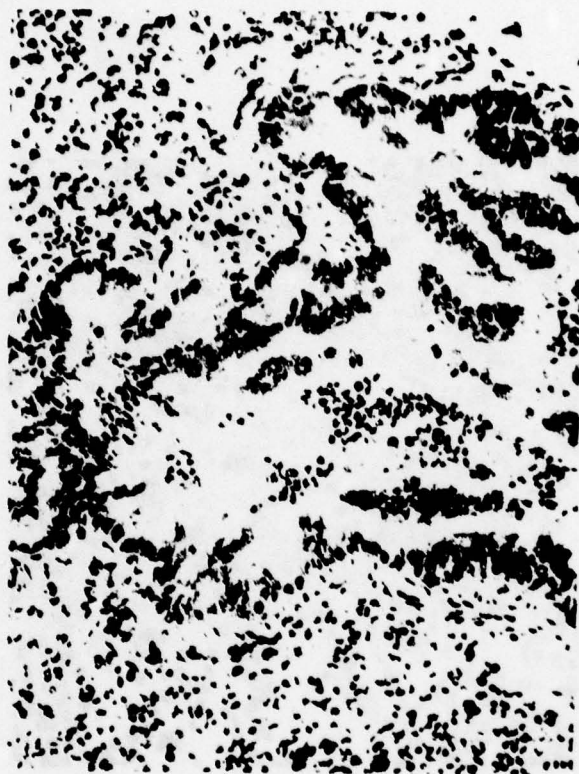


Fig. 6. Lung, guinea pig infected intra-tracheally with N. gonococcus, 12 hours post-challenge. The alveoli and bronchioles contain aggregates of neutrophils. Bacteria are not apparent. X250.

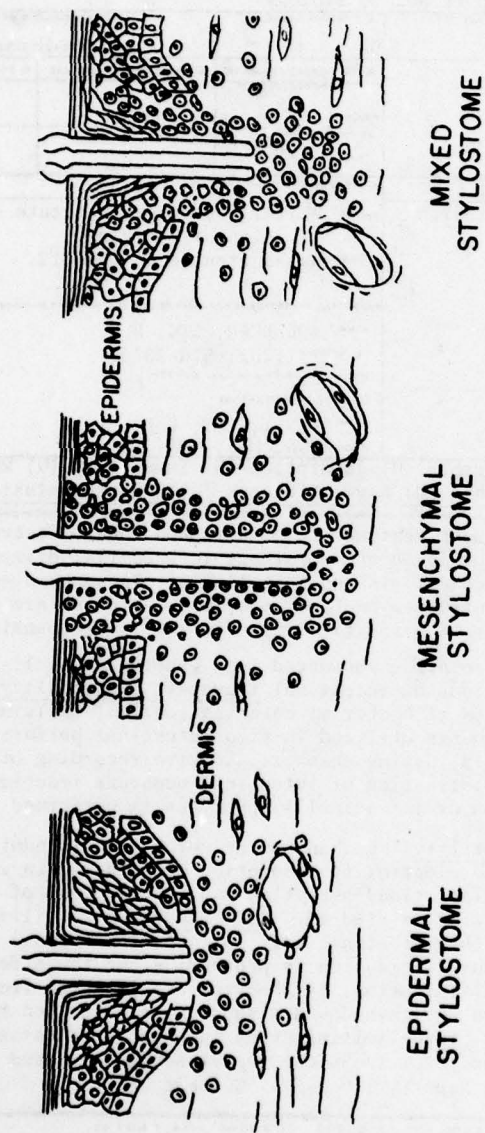


Fig. 7. Schematic drawing of the three types of stylostomes of Leptotrombidium spp. larvae. The epidermal stylostome of L. intermedium, the mesenchymal stylostome of L. fletcheri, and the mixed stylostome of L. arenicola and L. deliense.

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34. TECHNICAL OBJECTIVE, 35. APPROACH, 36. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.) 23(U) Research efforts in this department continue to be directed toward Gastrointestinal diseases of military importance. Focus is on enteropathogenic bacterial diarrheal disease including pathogenic E.Coli, Salmonellosis and Shigellosis. These have critical military relevance because of their influence on troop mobility. Studies are also performed on the determinants of fibrosis in parasitic liver disease (schistosomiasis). 24(U) Studies of bacterial diarrhea are being conducted in 3 general areas 1) Pharmacologic modification of effects of infections on intestinal transport and motility. 2) Intestinal cell membranes as determinants of bacterial colonization. 3) Cellular immune response to intestinal infection. Studies utilized in vivo intestinal perfusions of rabbits and rats, rat ileal loop models, Ussing chambers, in vivo recording of intestinal myoelectric activity and in vitro agglutination of intestinal membrane fractions. Isolation and functional characterization of intestinal lymphocytes is performed. 25(U) 77 10 - 78 09, Transport and motility - mechanisms by which methylprednisone and other drugs increase mucosal water and electrolyte absorption are studied in vitro. The effect of pressure and distention on intestinal secretion and the response of intestinal myoelectric activity to enteric infection is studied. Cell Membranes - utilizing a model of adherence of pathogenic E.Coli to the intestinal cell surface, the mucosal receptor for bacteria has been solubilized, characterization is proceeding and the effect of IgA on adherence is studied. Immunology - intestinal lymphocytes are isolated from animals with enteric infections their function determined, and a suppressor effect on these lymphocytes documented. Liver Fibrosis - rate limiting steps in hepatic fibrosis and their pharmacologic modification are studied. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.									

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE .

Work Unit 005 Gastrointestinal Diseases of Military Importance

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Description

The research activities in this Department have continued to focus on the toxigenic and/or invasive enteropathogenic diarrheal diseases caused by E.Coli, Salmonella, Shigella and, to a lesser extent, Cholera. Three basic sets of questions are being asked about the pathogenesis of these diseases.

a. Pathophysiology of Intestinal Transport - What enzymatic and hormonal mechanisms mediate bacterial-induced secretion of water and electrolytes by the intestine? What are the normal mechanisms for salt and water absorption? How do absorptive and secretory mechanisms interact? Do they share any pathways? Can pharmacologic agents increase salt and water absorption in the face of infection or decrease secretion induced by bacterial toxins?

b. Role of Host Immune Mechanisms - How do disease causing bacteria interact with the normal cellular (lymphocyte, macrophage, polymorphonuclear leukocyte) and humoral (antibody) immune defense mechanisms in the intestine? What are the most effective means of inducing active cellular and humoral immunity to enteric infection (i.e., how can an effective vaccine for enteric infection be produced? What factors (antibody, complement) enable lymphocytes to kill enteric bacteria in vitro? Are these factors of equal importance in an in vivo model of enteric infection?

c. Role of Intestinal Surface Characteristics - How do disease causing bacteria interact with the surface of the gastrointestinal tract? What characteristics of this intestinal

surface permit disease causing bacteria to adhere to and colonize the small intestine? What characteristics of the intestinal surface permit specific interactions with toxins produced by bacteria. Can specific interactions of bacteria or their toxins be altered by orally administered agents?

In addition, studies on liver fibrosis in schistosomiasis, which is the most prevalent form of serious liver disease worldwide, have been initiated. Liver fibrosis is that process that determines reversibility or progression to chronic illness in all liver disease and the key biochemical event in this process is the deposition of collagen. Studies in this Department have focused on collagen metabolism in liver tissue from two animal models of schistosomiasis as well as from patients with this disease.

Progress and Results:

A role for Na^+ - K^+ -activated adenosine triphosphatase (Na-K-ATPase) in intestinal sodium and water absorption under basal conditions has not been demonstrated in vivo. To delineate this role, adjacent rabbit ileal loops were simultaneously perfused in vivo with a Ringer- HCO_3 solution. The perfusate was then changed in one of the loops to a Ringer- HCO_3 solution containing ouabain. After the perfusion, each loop was assayed for Na-K-ATPase and Mg-ATPase activities. Perfusion with 19.2 mM ouabain produced net sodium and water secretion and a 32% reduction in Na-K-ATPase activity. Ouabain perfusion did not alter the activity of Mg-ATPase or affect histology. When a Ringer- HCO_3 solution containing 15 mM glucose was perfused, ouabain reduced glucose absorption by 50%. As predicted from the known competition between ouabain and potassium for a Na-K-ATPase receptor site in vitro, ouabain-induced intestinal secretion and Na-K-ATPase inhibition could be reversed with a Ringer- HCO_3 solution containing 25 mM KCl. These results suggest that: 1) mucosal Na-K-ATPase plays an important role in the ileal absorption of sodium, glucose, and water under basal conditions; and that 2) an underlying intestinal secretory process exists as revealed by the selective in vivo inhibition of intestinal absorption. (15)

Basic mechanisms of electrolyte transport in the rat ileum were defined using in vitro techniques. (1) The short-circuit current (Isc), potential difference (PD), tissue conductance (Gt), and Na and Cl fluxes in the short-circuit state across the rat ileum were studied in Ussing chambers using a variety

of bathing solutions. In HCO_3 -Ringer solution, net Na absorption was not significantly different from zero, net Cl secretion was observed, and I_{sc} exceeded the algebraic sum of net Na and Cl transport. Addition of 10 mM glucose increased I_{sc} , PD, Gt, unidirectional mucosa (M)-to-serosa(S) Na flux, and net Na absorption which accounts for mostly the increase in I_{sc} . Removal of HCO_3 from the bathing solution did not alter any of the transport parameters except decreased the unidirectional M-to-S Cl movement and increased net Cl secretion. 50% reduction of the Cl concentration in the HCO_3 -free solution decreased the net Cl secretion to the level of that in HCO_3 -Ringer solution and increased net Na absorption. In Cl-free solution, I_{sc} was only 50% of that in HCO_3 -Ringer solution and was equal to net Na absorption. The I_{sc} and PD were reduced to values near zero and net Cl transport was abolished by removing Na from the bathing solution. These results are consistent with the mechanism of electrolyte transport in the rat ileum that includes: an electrogenic Na absorption; and electrogenic Cl secretion; a neutral NaCl secretion; and a Cl absorption- HCO_3 secretion exchange system.

The effects of methylprednisolone administration on electrolyte transport in vitro rat ileum were next studied. (2) Administration of the glucocorticoid methylprednisolone, 3 mg/100 g of body weight for 3 days, in intact rats increased intestinal mucosal guanylate cyclase and Na-K-ATPase activities, short-circuit current (I_{sc}), potential difference (PD), net Na absorption and net Cl secretion and reserved HCO_3 transport (represented by a residual ion flux, J_R) from secretion to absorption in isolated ileal mucosa. At 6 hr after a single dose of methylprednisolone, the stimulation of guanylate cyclase activity was already maximal whereas Na-K-ATPase activity was not altered. The associated changes in intestinal transport properties with the increased guanylate cyclase activity were an increase in I_{sc} and PD and an inhibition of net Na and Cl absorption. These results suggest that an initial response to methylprednisolone administration is a persistent increase in intestinal guanylate cyclase activity that mediates an increase in I_{sc} and PD and an inhibition of Na and Cl absorption, then followed by a superimposed effect of increased Na-K-ATPase activity that mediates further increases in I_{sc} , PD, and net Na absorption. The third effect of methylprednisolone is the reversal of HCO_3 transport and its mechanism is not known.

he effects of methylprednisolone administration on the intestinal mucosal enzyme guanylate cyclase was also studied. (16) The role of the intestinal mucosal enzyme guanylate cyclase (GC) in intestinal water and electrolyte transport is unclear. Increased GC activity or cGMP content have been associated with both catecholamine induced increases in intestinal absorption and with heat stable E.Coli enterotoxin induced secretion. To further study the role of GC in intestinal transport, we examined the effect of methylprednisolone (MP) treatment on intestinal mucosal GC specific activity. Rats were injected subcutaneously with MP, 3mg/100 g b.w., and GC activity measured at varying times in whole homogenates of rat ileal mucosa by a modification of methods of Krishnan and Nesbitt. A significant increase in GC activity was observed as early as 6 hours after MP treatment ($1,144 \pm 107$ vs. 754 ± 64 p moles cGMP/mg protein/10 min, $p < 0.05$). This increase in GC activity progressively increased between 6 and 48 hours ($1,330 \pm 56$ p moles cGMP/mg protein/10 min) after MP injection and was not increased by further injections. Mucosal Na-K-ATPase specific activity was not increased until 16 hours following MP injection. These studies demonstrate that: 1) MP increases intestinal mucosal GC specific activity as early as 6 hours after treatment; 2) The MP induced increase in GC activity precedes the MP induced increase in Na-K-ATPase activity and may precede the MP induced increase in intestinal absorption of water and electrolytes. The functional significance of increased levels of intestinal mucosal GC in intestinal absorption remains to be determined.

It has been reported that the lactamimide RMI 12330A inhibits the cholera toxin (CT)-stimulated intestinal fluid hypersecretion and AC activity in the rat. Our studies were designed to further characterize the inhibitory effects of RMI 12330A. (17) The effects of RMI 12330A on the total ionic transport (represented by the short-circuit current, I_{sc} , by definition), electrical potential difference (PD), and tissue conductance (G) in stripped rabbit ileum bathed in Ringer's- HCO_3 solution were studied in Ussing chambers. Normal tissues and tissues treated with purified cholera toxin (50 ug/ml/15 cm loop) in vivo for at least 4 hrs were used. At 10^{-5} and $10^{-4}M$, RMI 12330A slightly decreased the I_{sc} and PD but did not change G. $10^{-3}M$ RMI 12330A abolished the I_{sc} and PD in approximately 30 min and increased G two to three fold in one hr. This effect was irreversible and the response of I_{sc} and PD to 10 mM glucose or theophylline was also abolished. Mucosal AC, Na-K-ATPase, and Mg-ATPase activities were measured with mucosal scrapings

incubated in the presence and absence of $10^{-3}M$ RMI 12230A as a function of time. The basal, fluoride-, and CT-stimulated AC activities were decreased rapidly and were eventually abolished in 50 min. Na-K-ATPase and Mg-ATPase activities were also decreased by RMI 12230A: respectively being abolished and inhibited more than 50% in 50 min. These results indicate that the action of RMI 12230A at $10^{-3}M$ has several effects: 1) inhibition of multiple intestinal enzyme systems and 2) abolition of active ion transport and destruction of tissue integrity as shown by the electrical data. Finally, the above would suggest that the use of RMI 12230A as an inhibitor of stimulated intestinal secretion is questionable.

It has previously been shown that *Shigella* produces not only the well known dysentery but also a watery diarrhea. While it has been demonstrated that this is due to a toxin - no thorough study of the toxin's effect on transport has been made. The procedure of these experiments (3) was to first inoculate a closed ileal loop with toxin. After four hours the segment was removed, washed with saline, the muscularis mucosae removed, and the tissue mounted in standard Ussing chambers. A loop from the same animal and inoculated with saline was prepared in an identical manner. The potential difference (Pd), conductance (G), short-circuit current (Isc), Na and Cl fluxes were measured.

The results showed no effect on the electrical parameters and secretion of Na and Cl. The unidirectional fluxes revealed that this was secondary to a movement of Na and Cl from the serosal to the mucosal side with no change in their mucosal to serosal movement. This is distinctly different from other secretagogues, i.e., cholera toxin, and *E. Coli* toxin, and suggests a new mechanism for stimulating secretion. Further studies are needed to more clearly define this mechanism.

Shigella dysenteriae is an organism capable of invading the bowel wall and may produce a toxin called *Shigella dysenteriae* I enterotoxin or Shiga toxin (ST). ST is distinctly different from the enterotoxin of *Vibrio cholera*. This laboratory has previously defined the presence of an organized complex, the migrating action potential complex (MAPC), in response to cholera toxin (CT). In the present study (18) we used myoelectric recording techniques to investigate and characterize the effects of ST on the small intestine. New Zealand white rabbits were used for all studies. The model consisted of an in vivo 12-cm ileal loop to which 4 Ag-Ag Cl electrodes were sewn to the

serosa of the small intestine above the ileal loop. A large catheter was inserted above the loop for outflow of luminal contents. A 1:300 dilution of ST was infused into the ileal loop at a rate of 0.15 ml/min. Repetitive bursts of action potentials (RBAP) and MAPC activity occurred in the ileal loop at a mean frequency of 4.5 and 1.1/hr, respectively. No fluid output from the distal loop catheter was noted. RBAP/MAPC activity was also observed in the proximal small intestine above the loop in response to the ST infusion at a mean frequency of 5.2 and 0.5/hr, respectively. The mean onset time for all activity was 3.5 hr. The following studies demonstrated that 1) ST induced 3 myoelectric responses, RBAP and MAPC activity; 2) both types of activity occurred within the infused loop and the noninfused small intestine above the ileal loop; 3) there was more RBAP activity than MAPC activity; and 4) myoelectric activity occurred in the ileal loop with no fluid accumulation. These studies suggest that the alteration in myoelectric activity may be responsible in part for the clinical syndrome of Shigella infection.

The mechanism of serotonin induced electrolyte secretion in rabbit ileum was studied in vitro. Serotonin has been implicated in the diarrhea of carcinoid syndrome and in vivo intestinal secretion has been shown to be induced by this agent in the rabbit. In these studies, (19) stripped, short-circuited tissues bathed by Ringer-HCO₃ solution were found either to spontaneously absorb net Cl from mucosa (M) to serosa (S) (Cl-absorbers) or to secrete net Cl from S to M (Cl-secretors). In the Cl-absorbers, 2.6×10^{-7} M serotonin creatinine sulfate (5-HTCS): 1) decreased M to S Na and Cl fluxes (J_{Na}^{ms} and J_{Cl}^{ms}) equally and decreased the tissue conductance (G), 2) reversed net Na and Cl fluxes (J_{Na}^{net} and J_{Cl}^{net}) from absorption to secretion, and 3) did not change the short-circuit current (Isc), PD, J_{Na}^{sm} , J_{Cl}^{sm} , or the residual ion flux (Jr). In Cl-secretors, 5-HTCS only increased PD and decreased G. 2.6×10^{-6} M 5-HTCS, in both Cl-absorbers and Cl-secretors: 1) increased J_{Na}^{sm} , J_{Cl}^{sm} , Jr, and PD, 2) decreased J_{Na}^{ms} , J_{Cl}^{ms} , J_{Na}^{net} , J_{Cl}^{net} , and G, and 3) did not alter Isc. At 2.6×10^{-5} M 5-HTCS, no effects were noted. These results suggest 1) the existence of Cl-absorbers and Cl-secretors in ordinary rabbits, 2) the presence of separate basal absorptive and secretory Cl transport processes, 3) that 5-HTCS inhibits a basal coupled NaCl absorptive process, and 4) that 2.6×10^{-6} M 5-HTCS stimulates a Cl and/or HCO₃ neutral secretory process.

The effect of altered intestinal water transport on rabbit ileal

blood flow was investigated. (20) A method is described for measuring ileal blood flow in the anesthetized (sodium pentobarbital) rabbit by the intraventricular injection of microspheres (15 μ m) labeled with 141 Cerium or 51 Chromium in which the amount of labeled microspheres lodging in the tissue is proportional to the blood flow. Blood flow to the ileal mucosa plus submucosa could be separated from flow to the ileal muscularis propria plus serosa by this method. Simultaneous and sequential injections of radiolabeled microspheres give similar measurements of ileal blood flow and microsphere injection did not affect ileal histology or ileal water absorption. Increasing ileal water absorption by treatment with the glucocorticoid methylprednisolone (3 mg/100 gm/day for 3 days) caused increased blood flow to both compartments of the ileum and also increased blood flow to the colon, liver, and kidneys; methylprednisolone treatment did not alter blood flow when studies were performed before the methylprednisolone-induced increase in ileal water absorption had occurred. In contrast, intestinal secretagogues which induced both active ileal water secretion (purified cholera toxin and serotonin) and passive ileal secretion (hypertonic mannitol) did not affect ileal blood flow. These studies indicate that increased ileal water absorption is associated with increased ileal blood flow while absorption is associated with increased ileal blood flow while intestinal secretion is not necessarily associated with an alteration in ileal blood flow.

Patients with intestinal obstruction can have intraluminal accumulation of fluid. Intestinal obstruction increases intraluminal hydrostatic pressure (IHP), however available information on the direct effect of IHP on both intestinal water and electrolyte transport in vivo remains incomplete. In this study (6, 21) ileal IHP was acutely increased in vivo in male New Zealand white rabbits, and the effect was determined on HOH nad electrolyte transport in the test loop and adjacent control loop using an invivo perfusion system with 14 C-polyethylene glycol as a nonabsorbable HOH marker. Unidirectional transport of HOH was determined using 3 HOH. Test loop IHP was increased by raising the efflux catheter 10, 20, or 30 cm above the abdominal surface, while control loop IHP was held constant at 0 cm HOH. Increased IHP caused a significant increase in test loop net HOH, Na, K, and Cl secretion and lumen to plasma and plasma to lumen transport of HOH, while transport in the control loop was not altered. Increased IHP was also associated with significantly increased loop length, diameter, and

lumen volume. Following transport studies mucosal adenylate cyclase activity and histology were assessed. Altering IHP did not significantly affect adenylate cyclase activity. No histological alteration occurred at IHP of 10 cm HOH. At 20 cm HOH submucosal and muscularis hemorrhages were visible. At 30 cm HOH histological alterations, including epithelial sloughing, were observed. In summary, increased IHP in rabbit ileum caused: 1) pressure dependent secretion of HOH and electrolytes and 2) increased unidirectional fluxes of water in lumen to plasma and plasma to lumen directions. This secretion was not associated with significant alterations in mucosal adenylate cyclase activity, was not always associated with histological alterations, and was not apparently caused by a systemic effect of luminal distention. Intraluminal accumulation of fluid seen in intestinal obstruction may be related to the secretion induced by increased IHP. Acutely elevated IHP does not alter a number of important active transport processes (5) including mucosal ATPase activity, adenylate cyclase activity or glucose mentation of water and electrolyte absorption but elevated IHP did alter passive transport (4) processes including unidirectional water transport and mannitol clearance. Mucosal and whole segment blood flow were not significantly changed by increased IHP.

In studies of *Shigella* toxin diarrhea, a unique effect on bile flow was observed. (22). To date, studies of the pathogenesis of diarrhea have concentrated almost entirely on the direct intestinal contribution to increase stool water and have largely ignored contributions from other GI organs. The present studies were prompted by the observation that the intestinal proximal to rabbit ileal loops inoculated with *Shigella dysenteriae* I enterotoxin (ST) 18 hr previously was distended far more than that proximal to saline (NS) inoculated loops. 18 hr after inoculation of distal ileal loops, volume flow from the GI tract proximal to the duodeno-jejunal junction was collected by gravity. Inoculation of several intestinal secretagogues caused an increase in proximal gut flow (ST produced 35.8 ml/2hr, choleragen (CE) 30 ml/2hr and hypertonic mannitol (HM) 30 ml/2hr) compared to NS inoculated loops (17 ml/2hr). To determine the source of this proximal luminal fluid, the common bile duct, pancreatic duct, duodenum just distal to the pylorus, and proximal and distal small intestine were separately cannulated in individual animals. Increased biliary flow occurred 18 hr but not 4 and 12 hr after distal ileal inoculation of ST (19.4 ml/2hr), CE (13.6 ml/2hr) and HM (16.3 ml/2hr) compared to NS (9.7 ml/2hr). No increase was

observed in pancreatic, gastric or proximal or distal small intestinal flow. The intestinal secretagogue-related bile flow had increased total bile salt secretion, total phospholipid secretion and an increased (BS)/(phospholipid), but had no change in bile salt concentration or composition or phospholipid concentration compared to bile from NS inoculated animals. These findings suggest that both active and passive distal ileal secretion in the rabbit indirectly increase bile flow. This choleresis: 1) is associated with increased biliary bile salt secretion; and 2) is associated with a lag time occurring significantly after the onset of the ileal secretion. These results suggest that distal ileal secretagogues could indirectly contribute to fluid loss from the GI tract through a choleric mechanism.

A clinical study was performed to investigate the mechanism of diarrhea production in a patient with chronic diarrhea (23). A 46 year old female is described who had a 13 1/2 year history of watery diarrhea associated with hypokalemia and hypochlorhydria. The diarrhea was secretory as measured by triple lumen tube perfusion and was associated with an elevated fasting plasma immunoreactive gastric inhibitory polypeptide (GIP) concentration of 750 pg/ml which stimulated to 4000 pg/ml after a standard meal. The diarrhea decreased after partial pancreatectomy. Diffuse pancreatic islet cell hyperplasia was present and while GIP was unmeasurable in the pancreas of normal subjects, it was at least 83 ng/gm wet weight in this patient. Postoperatively the patient's diarrhea responded dramatically to oral nicotinic acid.

It has been shown that it is possible for intestinal mucosa to grow across an adjacent serosal surface. While this tissue (neogut) appears histologically normal and has appropriate absorption enzymes, there have been no studies of its physiologic functioning. For our experiments (7) a longitudinal incision was made in the rabbit's ileum and then anastomosed to the colon such that the intestinal mucosa and the colonic serosa were approximated. After a period of 6-12 weeks, this tissue was removed, the serosal and mucosal tissues separated as thoroughly as possible and the neogut mounted in standard Ussing chambers. The conductance (G), the potential difference (Pd), the short-circuit current (Isc), and the Na and Cl fluxes were determined. The results showed a Pd of 1.3 mv/cm², a G of 7.38 mmho/cm², and Isc of 0.48 microeq/hr-cm². All of these values were significantly different from zero. Due to the thickness of the tissue, even after stripping, we were unable

to obtain accurate Na and Cl fluxes. The presence of these electrical parameters implies that the neogut is capable of functioning physiologically but at a reduced level from normal tissue.

The cecum of the germ-free rat is filled with a large volume of liquid: cecum plus cecal contents comprise up to 25% of the rodent's body weight. To explain the presence of the liquid cecal contents in the germ-free rat, cecal transport of water and electrolytes was studied using a closed loop technique (24) with ^{14}C -polyethylene glycol as a non-absorbable marker. When NaCl (154 mEq/L) was instilled into germ-free rat ceca, absorption of water, Na and Cl occurred and was similar to that in conventional animals. In contrast, when an equal volume of supernatant from germ-free cecal contents was instilled into germ-free rat ceca, secretion of water, Na and Cl occurred. Similarly, when supernatant from germ-free cecal contents was instilled into ceca of conventional rats, secretion of water, Na and Cl occurred at rates equal to that seen in the germ-free rat. Therefore, it appeared that the composition of the germ-free cecal contents was primarily responsible for the cecal secretion. Analysis of the germ-free cecal contents to determine what caused the cecal secretion revealed: very low measureable anions (Cl 2 , HCO_3 2 mEq/L); a low Na concentration; slight hyperosmolality as determined by freezing point depression and elevated colloid osmotic pressure. Fluid made up with Na_2SO_4 to resemble the germ-free cecal supernatant in ionic composition and absence of exchangeable anions produced cecal secretion equal to that of the germ-free rat cecal contents. Fluid simulated to reproduce the effects of the low Na concentration and also the colligative properties and colloid osmotic pressure had minimal effects on cecal transport. These studies demonstrated that cecal enlargement in the germ-free rat is associated with cecal secretion of water and electrolytes; that germ-free ceca can transport water and electrolytes in a normal fashion; and that cecal secretion in the germ-free rat is due primarily to the absence of exchangeable anions in the germ-free cecal contents.

Uncertainty remains regarding the significance and mechanism of changes in intestinal HOH and electrolyte transport induced by the B-adrenergic receptor blocker propranolol (P). These studies were designed to determine the effect of P on a well-defined intestinal secretory process. HOH and electrolyte secretion and increased adenylate cyclase (AC) activity

were observed in (CT) inoculated ileum and colon, but Na-K-ATPase, Mg-ATPase and cyclic nucleotide phosphodiesterase (PDE) were unaffected. When dl-P(4mg/kg) was injected daily for 3 days before loop inoculation, P did not affect HOH or electrolyte absorption or mucosal AC, Na-K-ATPase or PDE activities in saline inoculated ileal or colonic loops. However, dp-P significantly decrease CT-induced increases in AC in control and P treated animals were similar. D-P has the non-specific membrane-blocking effects of P but is not a B-blocker. Pre-treatment with d-p (4mg/kg) for 3 days did not affect CT-induced secretion. To document that the P inhibition of secretion occurred after AC activation, a model of ileal secretion induced by dibutyl cAMP (DB) was developed. Ileal perfusion with DB caused net HOH secretion in controls whereas in animals treated with dl-P (4mg/kg) for 3 days, DB-induced HOH secretion was significantly decreased. These results indicate that: 1) dl-P is able to inhibit CT-induced ileal and colonic HOH secretion in the rat; 2) this effect is not associated with inhibition of CT activation of AC and does not involve the non-specific membrane-blocking properties of P; 3) this inhibition apparently involves a step in intestinal secretion following AC activation. (25)

Early investigations of freezing and thawing murine spleen cells showed that such treated cells could protect lethally irradiated mice and were capable of antibody production. More recent work on murine cells indicated that thymus-derived lymphocytes (T-cell) were more susceptible to damage on freezing and thawing than were bursa-equivalent lymphocytes (B-cell). Damjanovic et.al. have shown that some plaque-forming cells (PFC) survive freeze drying, while Bohm and Dvorak were able to recover most plaque-forming cells after storage in liquid nitrogen for 37 weeks. We report (26) on experiments designed to investigate the ability of murine spleen cells to survive a single cycle of freeze-thawing. The recovery of plaque-forming cells was one method used to determine the functional capacity of frozen-thawed cells. Utilizing tissue culture systems, we were also able to measure both the ability of recovered cells to respond to an in vitro antigenic challenge and the blastogenic response of cells to mitogenic agents. Cultured frozen-thawed cells responded in vitro to an antigenic challenge of trinitrophenylated-bacteriophage T4 (TNP-T4), a T-cell independent antigen better than they responded to sheep red blood cells (SRBC), a T-cell dependent antigen. The uptake of (¹²⁵I)-iododeoxyuridine ((¹²⁵I) IUDR) after stimulation of cells with phytohemagglutinin-P (PHA-P), a mouse T-cell mitogen or lipo-

polysaccharide (LPS), a B-cell mitogen was severely decreased by a single cycle of freeze-thawing.

Calcium ionophore A23187 will induce human white blood cells to kill autologous red blood cells. Optimally, the assay is run overnight, at 25°C, at a 25 or 50 to 1 killer to target cell ratio, and at a final ionophore concentration of 2.5 ug/ml. WBC's which were frozen and thawed three times could still mediate ionophore induced cellular cytotoxicity. Using intact cells, granulocytes (67.2% cytotoxicity), monocytes (34.8%), B cells (22.0%) and Null cells (19.3%) were effector cells but T cells (7.4%) were not. When cell fragments were used, all cell types including T cells were able to effect cytotoxicity (33.5% to 84.5%). These studies may implicate a calcium-dependent, membrane-localized mechanism in cellular cytotoxic processes.(27)

The mitogenic potential of normal human peripheral blood lymphocyte subpopulations in response to the plant lectins phytohemagglutinin, and pokeweed mitogen has been examined. Doubly purified B-, Null-, and T-cell fractions were used by themselves and in conjunction with small percentages (1 to 10%) of other subpopulations. Purified human B and Null cells were found to be noneactive. However, when incubated with as little as 1% T cells, significant mitogenic responsiveness by B and Null cells was observed. This helper effect was apparent with both normal and irradiated T cells. It would thus appear that mitogenic responsiveness of unseparated mononuclear cells is predominantly a function of T cells either as responding cells or as necessary helper cells. (28)

A large proportion of DNA synthesized in vitro by human lymphocytes stimulated with plant mitogens or specific antigens is selectively excreted from the cells. To determine if DNA excretion differs among various types of lymphocytes, we examined purified human lymphocyte subpopulations for DNA synthesis and excretion in response to stimulation by L-PHA. The relative proportion of newly synthesized DNA that is excreted by unseparated mononuclear cells, macrophage-depleted cells, T, and B lymphocytes is identical despite great differences in the magnitude of their responses. Low levels of both DNA synthesis and excretion by macrophage-depleted cells and B cells can be increased by reconstitution with macrophages and T cells, respectively. These data indicate that DNA excretion is a general property of lymphocytes stimulated to undergo DNA synthesis by plant mitogens. (29)

The development of profound leukocytosis in a patient with leukemic reticuloendotheliosis (LRE) enabled us to obtain purified LRE cells for the investigation of their structural and functional characteristics. The LRE cells of our patient bore surface immunoglobulin and had complement receptors but did not bear F_c receptors and did not form rosettes with sheep erythrocytes. By electron microscopy, the cells were observed to contain typical ribosome lamella structures and to phagocytize both 0.81 μ m latex particles. They were adherent to both glass and nylon wool fibers. The mitogenic response to erythroagglutinating phytohemagglutinin was normal in magnitude but delayed chronologically. The binding of 125 I-labeled plant lectins was used to characterize the surface topography of LRE cells. Results of these studies indicated that the LRE cell surface differed significantly from the surface of normal T and B lymphocytes and chronic lymphatic leukemia cells. The LRE cells were capable of both stimulating and responding in a one-way mixed lymphocytes culture. However, the LRE cells were not active as effector cells of either cell-mediated lympholysis, a T cell function, or antibody-dependent cellular cytotoxicity, a null cell function. In contrast, they were effector cells of lectin-induced cellular cytotoxicity showing that they did possess the capacity to function as cytotoxic effector cells. These data indicated that the LRE cells in our patient had surface and functional characteristics of both lymphocytes and monocytes. (30)

Binding studies with 5 purified plant lectins were used to investigate membrane alterations in lymphocytes from patients with chronic lymphatic leukemia (CLL). Compared to normal human B lymphocytes, CLL lymphocytes had fewer receptors for E-phytohemagglutinin, wheat germ agglutinin, and concanavalin A, and more receptors for L-phytohemagglutinin. Receptors for Ricinus communis agglutinin were the same on both normal and CLL cells. Since the lectins bind to complex carbohydrates on the cell surface, these data suggested that the carbohydrate composition of CLL membranes differed from that of normal lymphocytes. To investigate this point, exposed membrane sialoglycoproteins on intact cells were radiolabeled by a combination of mild periodate oxidation followed by reduction with NaB^3H_4 . Analysis by SDS polyacrylamide gel electrophoresis of extracts from the radiolabeled cells indicated that the CLL cell membranes contained the same protein components as normal lymphocyte membranes, but that these components were generally less heavily glycosylated in CLL. However at least 3 CLL cell membrane proteins possessed more carbohydrate than the normal. To investigate

mechanisms responsible for the altered glycosylation of CLL cell membrane components, we examined glycosyltransferase activities in intact cells from CLL patients and normal donors. Compared to normal cells, CLL cells demonstrated decreased ability to transfer N-acetyl glucosamine from UDP-N-acetyl glucosamine to an appropriate exogenous glycoprotein acceptor. Likewise the ability of CLL cells to transfer N-acetyl neuraminic acid, galactose and N-acetyl glucosamine from their respective nucleotides to endogenous acceptors located intracellularly was reduced relative to normal. We conclude that CLL lymphocytes are characterized by a specific pattern of altered cell surface glycoproteins which can be detected by lectin binding studies. These alterations may be related to decreased glycosyltransferase activity in the CLL lymphocyte. (31)

Peripheral blood mononuclear cells from 49 Thai adults infected with either Plasmodium falciparum or Plasmodium vivax were examined in order to determine the percentage of T, B, and Fc receptor bearing cells present. In comparison to healthy controls, both the percentage and concentration of peripheral T cells were decreased in the malaria infected individuals as assessed by formation of rosettes with sheep red blood cells. The percentage of peripheral B cells was increased but their concentration was unchanged, as assessed by two techniques: the presence of surface immunoglobulin and the presence of a complement receptor. Both the percentage and concentration of lymphocytes bearing Fc receptors were unchanged in infected individuals. Finally, calculation of the changes in "Null" cells (defined either as non-T, non-B lymphocytes or as non-T, non-B, non-Fc receptor bearing lymphocytes) revealed an increase in "Null" cell percentage but a decrease in absolute number of "Null" cells. These data indicate that in adult Thai patients naturally infected with malaria, there is a real loss of circulating T lymphocytes with no real change in B, Fc receptor bearing, or "Null" lymphocytes. (32).

A 4 1/2-yr-old black boy was seen at 26 mo. with failure to thrive, recurrent pneumonia, and mucocutaneous moniliasis. Immunoglobulins were normal. Lymph node biopsy showed germinal centers but sparse paracortical cellularity. Peripheral blood lymphocytes formed 50% EAC rosettes and 6% E rosettes. He was lymphopenic and had absent responses to a battery of delayed hypersensitivity skin tests and dinitrochlorobenzene (DNCB). Red cell and lymphocyte ADA levels were 1% and 8% of normal respectively. With weekly thymosin injections, moniliasis cleared, peripheral E rosettes rose to 60%, and the delayed

skin test with diphtheria-tetanus (DT) became weakly positive. Lymphopenia and pneumonia persisted. ADA replacement by transfusion of fresh frozen plasma and irradiated red cells were added. The pneumonia cleared. After 2 yr of treatment, DT and streptokinase-streptodornase (SK-SD) skin test are positive, E rosettes are 40% to 50%, and total lymphocytes and phytohemagglutinin (PHA) response are low-normal. Bone marrow and Rebuck skin window cellularity have returned toward normal. Lymphocyte ADA levels remain deficient. DNCB still does not elicit a response. Clinically, the child has grown; he attends nursery school and handles infections without difficulty. Thymosin and ADA replacement therapy have been found safe and effective. (33)

Previous studies of the immune system in Whipple's Disease (WD) have shown minor reversible changes in serum immunoglobulins and a marked irreversible defect in cell mediated immunity shown by abnormal delayed cutaneous hypersensitivity and PHA induced ³H thymidine incorporation. Studies of cell mediated cytotoxicity have not been reported. We have recently seen a patient with untreated WD who had a positive response to PPD. Studies on his peripheral blood lymphocytes (PBL) in the absence of autologous serum revealed: A) Percentage of T, B, Null cells and macrophages were comparable to those of normal controls (NL). B) The ability of WD PBL to effect antibody dependent cellular cytotoxicity (ADCC) and spontaneous cell mediated cytotoxicity (SCMC) against various target cells was comparable to that of controls:

		<u>ADCC-%cytotoxicity</u>		<u>SCMC-%cytotoxicity</u>
		<u>CRBC target</u>	<u>K-562 target</u>	<u>K-562 target</u>
NL	PBL	33.3	59.7	45.6
WD	PBL	35.8	62.5	53.9

C) WD PBL undergo mitogenesis to both Concanavalin A (Con A) and pokeweed mitogen (PWM). The response to the latter was less than that of the control:

		MEDIA		CON A		PWM	
DAY:							
NL	PBL	38±10	45±7	7248±938	8451±1745	5690±1068	9727±1437
WD	PBL	51±14	42±4	8136±321	8587±213	2110±110	3480±99

D) Preliminary studies indicate that WD PBL may be capable of acting as both stimulators and responders in mixed lymphocyte culture. We conclude that: 1) WD PBL may contain normal percentages of T, B, Null cells and macrophages. 2) In the absence of autologous serum, WD PBL may be effectors of ADCC and SCMC, suggesting normal function of Fc bearing cells. 3) In the absence of autologous serum, WD PBL may be capable of normal mitogenic responses, depending on the mito-en tested. (34)

Cimetidine has been shown to alter lymphocyte function in histamine stimulated animal systems. We studied 9 PTS with acid peptic disease immediately prior to, after 2-3 wks and after 6-8 wks of treatment with cimetidine. There were 7 normal controls (C) not taking cimetidine. After CBC with differential, PBL obtained by ficoll hypaque separation were analyzed for surface characteristics, cytotoxicity and mitogenesis. Cytotoxic and mitogenic assays were performed in paired media, one containing the subject's own serum *HS), the other containing fetal calf serum (FCS). PTS pretreatment WBC counts ($x=10100 \pm 989$) and monocuclear cell counts ($x=3404 \pm 240$) were significantly higher ($p < 0.01$) than those of C ($x=6100 \pm 491$; 2252 ± 207 respectively) and did not change in the interval studies. Surface characteristics (% cells esterase and surface immunoglobulin positive, E, EA and EAC rosette forming) did not differ between groups of during the treatment period. Percent spontaneous cell mediated cytotoxicity (SCMC) to K562 cells was depressed in PTS prior to treatment and returned to normal after 2-3 weeks of treatment. There was no difference between assays in HS and FCS.

Control	Pretreatment	P	2-3 weeks	P	6-8 weeks	P
38.4 ± 4.2	17.1 ± 2.0	0.01	26.2 ± 5.9	NS	36.8 ± 4.4	NS
38.6 ± 4.6	22.0 ± 4.8	0.01	42.3 ± 8.3	NS	43.8 ± 6.2	NS

Antibody dependent cellular cytotoxicity to chick RBC and K562 cells, lectin induced cellular cytotoxicity to human RBC, and mitogenesis to PHA, Con A and PWM did not differ between PTS and C before or during treatment or with assay media. We conclude: 1) Short term cimetidine therapy induced no significant abnormalities in immunologic tests studied; 2) PTS with acid peptic disease have elevated cell counts and suppressed SCMC; 3) Treatment with cimetidine is associated with return to normal of SCM persistently elevated cell counts. (8)

Colonization of the intestine by some enteropathogenic bacteria may be related to their ability to adhere to the mucosal surface.

We studied the ability of a non-invasive, pillated, type O-15 *E. Coli* which causes diarrhea in rabbits (RDEC-obtained from Dr. J.R. Cantey) to attach to isolated brush borders (BB) and microvillus membranes (MVM). RDEC has been shown by light, immunofluorescent and electron microscopy to colonize the intestinal mucosal surface of young rabbits. To test for *in vitro* adherence, RDEC *E. Coli* were incubated with rabbit BBs ($1-2 \times 10^7$ /ml) and observed by phase contrast microscopy. After 30 min at RT, adherence at high bacterial concentrations (10^9 bact./ml) was demonstrated by large aggregates of BB and bacteria. At 3×10^7 bact./ml, BB aggregation was rare but adherence of 3-6 organisms/BB was seen. Incubation at 40 delayed adherence. Ca^{++} and Mg^{++} ions were not required. Formalin treatment of BB prevented RDEC adherence. No adherence to rabbit BB was noted using a variety of nonpathogenic or pathogenic strains isolated from humans. Conversely, RDEC did not adhere to BB from G.Pig or human intestine. To further localize the mucosal receptor for RDEC to the intestinal surface membrane, rabbit MVM vesicles were prepared and incubated with RDEC. In a microtiter plate assay, distinctive ring patterns of agglutination of RDEC was observed using 10^8 bacteria and as little as 10 ug of MVM protein. No agglutination of control bacteria occurred in this assay. Neither reaction was inhibited by a variety of monosaccharides, (mannitol, D or L-fucose, galactose), sialic acid or amino sugars. In conclusion: 1) Intestinal colonization by RDEC correlates with *in vitro* adherence to rabbit, but not human or G.Pig, BB and MVM. 2) A sensitive microtiter assay for MBM agglutination of adherent bacteria has been developed which may prove useful in screening enteropathogens for the ability to adhere to the intestinal mucosa. (9)

Adherence of pathogenic bacteria to the intestinal brush border (BB)/microvillus membrane (MVM) surface may permit organisms to colonize the bowel. We studied the ability of a strain of *E. Coli* (RDEC) enteropathogenic for rabbits to adhere to rabbit BB *in vitro*, and then attempted to solubilize the epithelial receptor from rabbit MVMs. When 10^5 BB were mixed with 10^7 bacteria in 100 ul for 15 min. @ 23°C, 11.8 RDEC adhered per BB compared to 1 bacteria per BB for a nonpathogenic strain of rabbit *E. Coli*. Receptor activity could be solubilized from the MVMs by detergent treatment (Triton-X-100) or proteolytic digestion (papain). MVMs were incubated in the presence of 0.25% Triton-X-100 for 1 hr., then centrifuges at 100,000g for 1 hr. The dialyzed supernatant was concentrated to 1 mg/ml. This supernatant agglutinated RDEC at levels of 100 ug/ml. 12 proteins bands ranging from 30,000 to 100,000 daltons were

detected by SDS-polyacrylamide gel electrophoresis (PAGE). RDEC receptor activity was also recovered in a 30,000g supernatant after papain digestion of rabbit BBs. Again agglutination of RDEC was detected at a level of 100ug/ml. On SDS-PAGE, the papain digest consisted of 4 major bands of 103, 93, 36.5, 33.5×10^3 daltons M.W.. In summary, an intrinsic MVM component, mediating bacterial adherence to the intestinal mucosal surface, was solubilized by triton-X-100 or by papain (10, 35).

Colonization of the small bowel by some pathogenic strains of Escherichia Coli maybe mediated by specific adherence to the intestinal mucosal surface. In the present study, a quantitative in vitro assay was developed in order to characterize the adherence of RDEC-1 to RBB was clearly distinguishable under phase microscopy from nonpathogenic control strains of E.Coli; the average number of RDEC-1 per RBB was 11.5 ± 0.7 (SD) compared to 2.7 ± 0.4 and 0.8 ± 0.1 for the two control strains, HS and 640. Maximal adherence of RDEC-1 to RBB was reached within 15 minutes. RDEC-1 binding to RBB was temperature dependent with the rate of adherence at 37°C being significantly faster than at 40°C. Optimal adherence was seen between a pHs of 6.5 - 7.0 and dramatic inhibition of RDEC-1 binding occurring as the pH dropped below pH 5.0. RDEC-1 adherence was also sensitive to the electrostatic environment of the reaction mixture with these being a linear depression of adherence with increasing ionic strength up to 708m or KCl. Unlike other pathogenic strains of E.Coli, RDEC-1 adherence was found to be resistant to D-mannose and its analogs. Furthermore, RDEC-1 was unable to hemoagglutinate rabbit, guinea pig or human erythrocytes. In summary, in vitro adherence of RDEC-1 to RBB has been partially characterized however further investigation of the model is needed in order to determine the molecules basis of adherence and colonization seen in bacterial diarrhea. (11)

Development of a host immune secretory immunoglobulin A (I-SIgA) response to a pathogenic organism may assist in the initial clearing of bacteria from the intestinal tract and protect against re-infection by preventing mucosal adherence. We tested the ability of rabbit I-SIgA raised against an adherent, pathogenic E.Coli to block adherence of that organism to brush borders (BB) in vitro. I-SIgA was obtained from the colostrum of rabbits immunized by the orogastric route with RDEC-1 E.Coli to block adherence of that organism to brush borders (BB) in vitro.

I-SigA was obtained from the colostrum of rabbits immunized by the orogastric route with RDEC-1 E. Coli, a piliated type 0-15 adherent strain pathogenic for rabbits. The I-SigA had a titer of 7,490 agglutinating units (AU)/mg (1 AU agglutinates 10^8 bacteria). A similar I-SigA preparation administered orogastrically to rabbits in previous studies delayed intestinal colonization with RDEC-1. We incubated 10^7 bacteria with I-SigA in increasing concentrations, dispersed the mixture by vortexing, added 10^5 BB and continued incubation in a volume of 100ul for 15 min. The number of organisms adhering per BB progressively decreased as the concentration of I-SigA was increased: 11.8 ± 0.5 (1 S.D.) bacteria adhered per BB without I-SigA; 50% inhibition of adherence was noted with .78ug I-SigA/100ul. In further experiments, the adherent properties of RDEC-1 were transferred to a non-adhering, non-piliated Shigella species. A resulting strain (D-1-5) possessed pili and adhered to rabbit BB in a manner similar to RDEC-1. The I-SigA prepared against RDEC-1 also inhibited adhesion of D-1-5 to rabbit BB in vitro. These studies show that specific anti-RDEC-1 I-SigA inhibits both adherence of RDEC-1 and of the adherent, piliated product (D-1-5) of the mating of RDEC-1 with Shigella, to rabbit BB in vitro. The data suggest that inhibition of adherence may occur through the interaction of I-SigA with the bacterial surface structures (pili) responsible for mucosal adherence. (12,36)

An enteropathogenic E. Coli RDEC-1 (015:MN) has been found to cause a fatal diarrheal disease of young rabbits. This organism multiplies in the ileum and colon by adhering to the mucosal surface. RDEC-1 has also been shown to adhere in vitro to isolated rabbit brush borders and microvillus membranes. The present study investigated the specificity of this adherence phenomena both in vitro and in vivo. In order to determine regional specificity, ileal and jejunal RBB were prepared in parallel from feru rabbits. The mean number of RDEC-1 attached to the jejunal RBB was 6.0 compared to 11.5 RDEC-1 per ileal RBB (p .05). In addition the failure of RDEC-1 to adhere to rabbit erythrocyte and bladder cells indicated tissue specificity of RDEC-1 adherence. The ability of RDEC-1 to adhere to BB from different animals, i.e. rats and guinea pigs, was found to be essentially zero, also indicating that in vitro adherence of RDEC-1 with in vivo colonization of the entestinal tract rats, rabbits and guinea pigs were orally inoculated with either RDEC-1, a control E. Coli strain (HS) or sterile culture medium. Animals were incubated

for a period of 10 days during which the animals were checked daily for diarrhea, at sacrifice, *E. Coli* growth in jejunum, ileum and caecum was quantitated and serologically typed for the presence of RDEC-1 or HS. Of all the animals, only the RDEC-1 fed rabbits came down with diarrhea. Their ileums and caecums were colonized with a mean of $10g_{10}$ 8.35 and 9.07 RDEC-1 per g wet wt. of tissue, respectively. Negligible colonization was seen in any of the intestinal segments in either the HS or control fed rabbits. Similarly, only minimal growth of RDEC-1 or HS was seen in any bowel segment in the guinea pigs and rats. In summary, adherence of RDEC-1 in vitro demonstrates regional, tissue, and species specificity. Species specificity is also displayed in the ability of RDEC-1 to colonize the small bowel in vivo. The degree of specificity possessed by RDEC-1 suggests that there maybe a receptor located on rabbit intestinal epithelial cells that interacts specifically with RDEC-1 and promotes adherence and colonization. (13)

Studies of human liver biopsy specimens completed shortly after transfer of these activities to WRAIR (37,38) showed that liver collagen content is increased 2 to 6-fold over normal, and that there are increases in all three of the specific collagen types present in normal liver (collagen Types I, III, and IV). Studies of synthesis of collagen measured in human liver slices by quantitative methods for the first time showed a 4 to 25-fold increase in this process compared with that in normal liver. The level of free proline, an important amino acid in the regulation of collagen synthesis, was increased in fibrotic liver, and the source of this increased proline was found to be arginine.

Investigation of the conversion of arginine to proline in mice with *Schistosoma mansoni* infection confirmed that this was the preferred pathway in comparison to conversion from the alternative proline precursor, glutamic acid (39). These results were consistent in experiments with liver slices and tracer studies with living mice.

Study of experimental inhibitors of proline hydroxylation in the mouse model system showed that several of these compounds selectively blocked this critical step in collagen biosynthesis in liver slices, and suggested the possibility of their use in living animals.

Development of a new animal model systems that more closely

resembles chronic human schistosomiasis was begun by studying rabbits at 6 months and 1 year after infection with S. japonicum. Initial results suggested that liver histology, collagen content, collagen synthesis rates, and metabolism of key precursor amino acids of collagen were all consistent with earlier data from human tissue, establishing this model as the preferred system for our current studies of biochemical regulation, prevention, and potential pharmacologic reversal of the process of liver fibrosis. (14)

A review on the subject of hepatic fibrosis was completed. (40).

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 005 Gastrointestinal Diseases of Military Importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION		2. DATE OF SUMMARY		REPORT CONTROL SYMBOL	
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				NAME: Macy-Mahady, Carol, Dr.					
25. REVIEWER (Precede with Security Classification Code)									
(U) Vaccines; (U) Epidemiology (U) Rickettsial infections; (U) Laboratory diagnosis;									
26. SYNOPSIS (Precede with Security Classification Code)									
23. (U) 1. Development of experimental rickettsial immunogens; 2. Pathology of rickettsial infection in laboratory animals; 3. Determination of the sequence of events leading to immunity following vaccination or infection. These studies are directly aimed at development of safe inactivated vaccines for protecting troops in the field and for development of accurate and sensitive tests to assay the extent of immunity induced by vaccination.									
24. (U) 1. Gamma irradiation of rickettsiae to produce attenuated, non-replicating organisms to be used as immunogens; 2. Determination of the stability of rickettsiae and gamma irradiated immunogens in the thawed and frozen states; 3. Evaluation of different strains and combinations of strains for use as gamma irradiated immunogens to provide long-lasting immunity against a broad spectrum of strains of Rickettsia tsutsugamushi; 4. Determination of the genetic basis of resistance and sensitivity of the mouse model to scrub typhus infection using specially bred mouse strains; 5. In vitro analysis of the effects of lymphocytes and lymphocyte products on macrophages.									
25. (U) 77 10 - 78 09 1. The thermal stability of gamma-irradiated scrub typhus immunogens has been established, and the C3H mouse model has been used to establish the optimum combination of strains for use as an immunogen to provide long-lasting immunity effective against a broad spectrum of challenge strains. 2. Current work has shown that susceptibility to scrub typhus infection can be regulated in the mouse by a single gene on chromosome 5. Studies are in progress to determine the mechanism of this resistance. 3. Serum from scrub typhus immune mice was capable of reducing the number of rickettsiae-infected normal macrophages by 50 percent. Rickettsial-stimulated spleen supernatants appeared to inhibit both cell entry and intracellular growth of rickettsiae in normal macrophages. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.									

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 006 Rickettsial Diseases of Military Personnel

Investigators.

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Associates: SP5 John A. Hallam; SP5 Denise G. Caron; SP5 Brian Weatherly

Description.

Studies on the mechanisms of rickettsial pathogenicity and of host resistance to infection have continued. Research efforts were concentrated into three main areas: (1) scrub typhus vaccine development, (2) host immune response to R. tsutsugamushi infection and vaccination, and (3) natural resistance to scrub typhus.

Progress.

I. Development of an inactivated scrub typhus immunogen.

A. Effects of temperature on the stability of Gamma-irradiated scrub typhus immunogens.

This laboratory recently reported on the efficacy of scrub typhus immunogens prepared by exposing infected 20% yolk sac suspensions of R. tsutsugamushi to 300 Krad of gamma radiation (1). In that study, the rickettsiae were frozen and stored at -70C until irradiated. They were then thawed rapidly at 37C and used immediately. This regimen was used because we had not established the effect of gamma radiation on the thermostability of scrub typhus rickettsiae; organisms noted for their instability. Therefore, in this study, we have examined the effects of prolonged frozen storage and of brief thawed periods on the stability of the immunogens. These results were contrasted with the effects of the same conditions on the lethality of the unirradiated organisms.

Assays were performed in female BALB/c mice, 18 to 22 g, using intraperitoneal (i.p.) injection in a standard volume of 0.2 ml for immunization and challenge. Immunogens and homologous challenge suspensions were prepared from infected yolk sacs of the Karp strain of R. tsutsugamushi following procedures previously

described (1). Yolk sac preparations of the Kato strain were used for heterologous challenge. Animals were observed for deaths occurring 6-28 days post-challenge, and 50% end points were calculated from the mortality data. Immunogen concentration was expressed in 50% mouse lethal dose (MLD_{50}) units based on the MLD_{50} titer of the suspension before irradiation.

The capacity of 300 Krad gamma-irradiated rickettsiae to induce effective immunity was assessed after frozen storage at both $-70^{\circ}C$ and $-20^{\circ}C$. Using a vaccination regimen that had been shown previously to provide excellent protection to mice (1), injections of 1.6×10^8 MLD_{50} units of stored immunogen were administered on days 0, 5, and 10. On day 31, challenge preparations of Karp and Kato were titrated in immunized and control animals. This schedule was repeated after storage for 3, 6, and 12 months.

The results (Table 1) indicate that there was no significant decrease in the capacity of irradiated organisms to confer protection against homologous challenge after storage at $-70^{\circ}C$ for 12 months. In addition, no significant decline in the lethality of unirradiated Karp was observed during this period. However, immunity indices for Kato challenge, which remained relatively constant for the first 6 months, declined 25-fold by 12 months. The adverse effect of storage at $-20^{\circ}C$ became evident more rapidly. The immunity index for homologous challenge decreased 100-fold after 3 months storage, and was reduced by approximately 10^4 after 12 months. The ability to elicit heterologous protection waned very rapidly at this temperature, being undetectable after 3 months storage. The titer of the virulent rickettsiae stored at $-20^{\circ}C$ also declined rapidly during the first 3 months of storage. However, a small percentage of the organisms were able to retain viability, as no further reduction in MLD_{50} was observed at 6 months. Thus, it appeared that a fragile component in the irradiated immunogen responsible for heterologous (Kato) protection may have undergone slow deterioration during storage at $-70^{\circ}C$, and that the rate of deterioration was increased markedly by storage at $-20^{\circ}C$.

We also assayed the stability of immunogens at $4^{\circ}C$ and $37^{\circ}C$. Because extracellular scrub typhus organisms are quite labile at $37^{\circ}C$, the observation period was limited to 8 hr. We used the 50% protective dose (PD_{50}) assay (1) to test for immunogen deterioration. This procedure involved a single injection of immunogen diluted in 10-fold increments followed by a 10^3 MLD_{50} challenge at 24 days. Calculations from subsequent mortality data yielded the number of MLD_{50} units required to protect 50% of vaccinated mice against the standard homologous or heterologous challenge.

Table 1. Effect of temperature on stability of gamma-irradiated Karp immunogens and unirradiated rickettsiae during frozen storage.

Months of storage	Immunity index ^a				Log ₁₀ MLD ₅₀ /g yolk sac ^b	
	Karp challenge		Kato challenge		-70 C	-20 C
	-70 C ^c	-20 C	-70 C	-20 C		
0	6.0(±0.4) ^d	5.6(±0.4)	4.4(±0.3)	3.0(±0.4)	-8.9(±0.2)	-5.9(±0.3)
3	6.6(±0.4)	3.6(±0.4)	4.8(±0.5)	0.2(±0.2)	-8.5(±0.3)	-3.9(±0.2)
6	5.4(±0.5)	3.0(±0.5)	4.2(±0.5)	0.0(±0.3)	-8.3(±0.2)	-3.9(±0.2)
12	5.0(±0.6)	1.2(±0.6)	2.8(±0.6)	ND ^e	-8.2(±0.0)	ND

^a Immunity index = log₁₀ MLD₅₀ in vaccinated mice - log₁₀ MLD₅₀ in control mice.

^b MLD₅₀ titrations were performed 10 days after initiation of vaccination.

^c Storage temperature.

^d Value (± standard deviation).

^e Not done.

A comparison of the stability of immunogens and virulent rickettsiae at 4C and 37C is shown in Table 2. At 4C, the temperature normally used for short term maintenance of biologicals during experimentation, both PD_{50} for homologous challenge and MLD_{50} of virulent rickettsiae remained constant for 8 hr. The PD_{50} for heterologous challenge was unaffected at 4 hr, but exhibited a modest rise by 8 hr, indicative of a decrease in potency of the immunogen. At 37C, the ability of the immunogen to elicit homologous immunity was reduced approximately 40-fold within 4 hr, and the titer of virulent rickettsiae was depressed similarly. However, the protective capacity of the immunogen against Kato challenge was abolished. After 8 hr at this temperature, the lethality of the unirradiated organisms was also abolished, but no further change in homologous PD_{50} was apparent.

In these studies, unirradiated *R. tsutsugamushi* were stable during storage for 12 months at -70C or for 8 hr at 4C. Gamma-irradiated immunogens were slightly less stable at these temperatures, and could be maintained only for 6 months at -70C and for 4 hr at 4C without loss in immunogenicity. Immunogen stability monitored by both assays indicated that at least two components were required for full potency, and that the one required for stimulation of immunity to Kato challenge was more labile at each temperature than was the component which elicited homologous immunity.

B. The initial phase of the immune response after vaccination with gamma-irradiated scrub typhus immunogens.

In the annual report for FY 77 we noted that immunity to homologous strain (Karp) challenge developed quite rapidly, being at maximum level when first tested on day 17, 7 days after the third injection of 300 krad gamma-irradiated Karp immunogen. At that time, the mice were also resistant to challenge with 10^4 MLD_{50} of Kato, a heterologous strain of *R. tsutsugamushi*. Subsequently, we have tested further the rapidity with which an effective immune response is induced by challenging the mice at successively earlier times within the vaccination regimen. For clarity, the results depicted in Fig. 1, have been integrated with the results of the previous long term study. Thus, resistance to homologous challenge developed quite rapidly, being effective against a 10^4 MLD_{50} challenge 5 days after the first injection of immunogen and reaching peak level, as noted above, 7 days after completion of the vaccination regimen. However, immunity to heterologous challenge developed more slowly. Significant resistance to Kato was not achieved until day 17, when the animals were protected against 10^4 MLD_{50} of Kato. Since

Table 2. Effect of temperature on stability of gamma-irradiated Karp immunogens and unirradiated rickettsiae after thawing.

Hours after thawing	Log ₁₀ PD ₅₀ ^a		Log ₁₀ MLD ₅₀ /g yolk sac	
	Karp challenge		Kato challenge	
	4 C ^b	37 C	4 C	37 C
0	5.8(±0.4) ^c		6.6(±0.4)	-8.4(±0.3)
4	5.8(±0.4)	7.4(±0.4)	7.0(±0.4)	≥8.2(±0.4)
8	6.0(±0.4)	7.8(±0.4)	7.6(±0.4)	≥7.8(±0.4)
				-8.4(±0.2)
				≥-0.6(±0.3)

^a 50% protective dose, the number of MLD₅₀ units of immunogen required to protect 50% of vaccinated mice against a 10³ MLD₅₀ challenge.

^b Incubation temperature.

^c Value (± standard deviation).

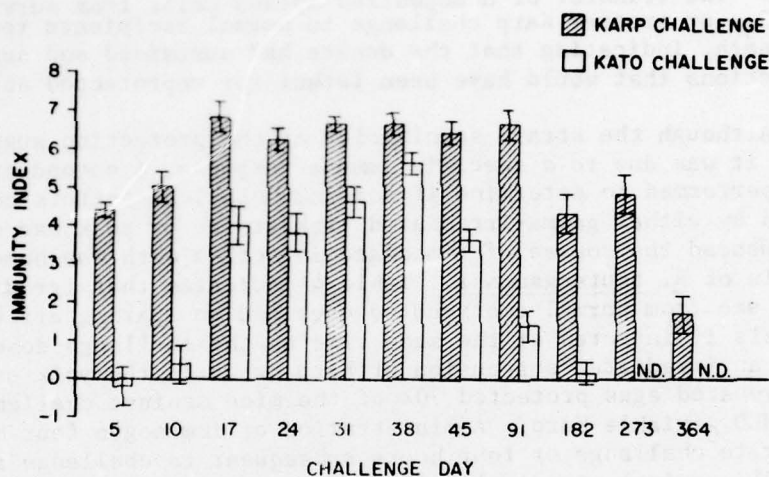


Figure 1. Development and duration of homologous and heterologous immunity achieved by vaccination with gamma-irradiated Karp. Vaccination regimen consisted of 3 i.p. injections of 1×10^8 MLD₅₀ units of immunogen given at 5 day intervals. Vertical brackets delimit \pm standard deviation.

homologous protection was substantial 5 days after the first immunogen injection, it was of interest to determine the earliest time at which the gamma-irradiated rickettsiae induced a protective response. In the initial experiment, standard suspensions of Karp and Kato were each diluted in irradiated Karp immunogen and titrated in mice. The results, shown in Table 3, indicated that the response induced by a single injection of immunogen was capable of masking the presence of 200 MLD₅₀ of virulent Karp, but as expected from the 5 day and 10 day heterologous challenge results (Fig. 1), did not alter the lethality of the Kato suspension. The transfer of homogenized spleen cells from survivors of 2 MLD₅₀ or greater Karp challenge to normal recipients resulted in death, indicating that the donors had sustained and survived infections that would have been lethal for unprotected animals.

Although the strain specificity of the protection suggested that it was due to a specific immune response, a second experiment was performed to determine if non-immunological factors contributed by either gamma-irradiated rickettsiae or yolk sac debris influenced the course of immediate infection with the homologous strain of *R. tsutsugamushi*. Table 4 indicates that irradiated yolk sac from normal embryonated eggs had no sparing effect on animals if injected at the same time as the challenge dose, but that an irradiated suspension of Karp grown in the yolk sac of embryonated eggs protected 70% of the mice against challenge with 500 MLD₅₀ viable Karp. Administration of immunogen four hours prior to challenge or four hours subsequent to challenge resulted in 60% survival of animals. Again, homogenized spleen cells from the survivors were lethal for normal recipients indicating that the donors had sustained active infections.

The rate of development of immunity to homologous challenge after i.p. vaccination was unexpectedly rapid. The results indicated that the immunogen generated a rapid, specific immune response capable of protecting mice against simultaneous homologous challenge. Specificity of resistance was demonstrated by the unaltered lethality of a simultaneous Kato challenge. However, a number of years ago, Edsall (2) noted reports of investigators who had observed protection against challenge with other infectious organisms or toxins administered concurrently with, or shortly after vaccination. In some instances early immune responses were implicated, although in others the protection appeared to be due to interference between vaccine and challenge doses for receptor sites, emphasizing the importance of incorporating proper controls to reveal contributions by non-immunological factors. For this reason, we tested the effects of irradiated normal yolk sac and the sequence of administration of challenge and vaccination doses.

Table 3. Survival of mice receiving simultaneous administration of immunogen and rickettsial challenge.

Challenge strain	No. survivors/No. vaccinated mice challenged ^b			Log ₁₀ MLD ₅₀ in vaccinated mice ^c		Log ₁₀ MLD ₅₀ in control mice ^c		Immunity index ^d
	2000 ^b	200	20	2	0.2			
Karp	ND ^e	10/10	10/10	9/9	10/10	≥ -6.2	$-9.0(\pm 0.3)$	≥ 2.8
Kato	0/10	1/10	0/10	3/10	9/10	$-8.1(\pm 0.2)$	$-8.2(\pm 0.2)$	$0.1(\pm 0.3)$

^a Mice were vaccinated with 1×10^8 MLD₅₀ units of irradiated Karp in a single injection i.p.

^b Number of MLD₅₀ in challenge dose as determined by titration in control mice.

^c Values (\pm standard deviation) are based on dilution factors used to achieve the challenge doses noted.

^d Immunity index = $\log_{10} \text{MLD}_{50}$ in vaccinated mice - $\log_{10} \text{MLD}_{50}$ in control mice.

^e ND = not determined.

Table 4. Effect of sequence of administration of immunogen and homologous challenge on mouse survival.

0 ^a	Sequence and time of injection		No. survivors/No. challenged
	4	8	
	Irradiated normal yolk sac ^b + Challenge ^c		0/10
	Immunogen ^d + Challenge		7/10
Immunogen	Challenge		6/10
	Challenge	Immunogen	6/10

^a Time of injections in hours.

^b 20% suspension of normal yolk sac irradiated with 300 Krad gamma radiation.

^c Challenge dose was 500 MLD₅₀ of viable Karp, determined by separate titration in control mice.

^d Immunogen consisted of 1×10^8 MLD₅₀ units of irradiated Karp.

Irradiated normal yolk sac did not modify the lethality of the Karp challenge, and it was not likely that resistance was due to an interference phenomenon as administration of challenge either before or after injection of immunogen resulted in protection similar to that achieved when the two injections were administered simultaneously. Further, homogenized spleen cell transfers indicated that the animals had survived infection with fully virulent rickettsiae which remained harbored in the spleens of the immune survivors.

II. Growth of R. tsutsugamushi in peritoneal macrophages of mice.

The central role of the macrophage as an effector cell of both humoral and cellular immunity has been well documented. Macrophages have been shown to play an important part in immunity to several obligate intracellular parasites and bacteria; however, the immunologic response arising during infection which facilitates the destruction of parasites or bacteria by macrophages varies with the invading organism.

Cellular immunity appears to play a role in heterologous strain immunity during scrub typhus infection of mice (3); the contribution of antibody in this system is less defined, and may play a more prominent role late in infection. Histopathologic studies of experimental murine scrub typhus demonstrated the presence of rickettsiae in peritoneal macrophages of lethally infected mice, but not in mice which survived infection and were immune (4).

The purposes of this study were to: (1) evaluate the interaction of R. tsutsugamushi and BALB/c peritoneal macrophages in vitro, and (2) to determine the immunological factors which enhance the intracellular degradation of R. tsutsugamushi in professional phagocytes.

A. Growth of scrub typhus rickettsiae in peritoneal macrophages.

Resident peritoneal macrophages from BALB/c mice supported the growth of R. tsutsugamushi, strain Gilliam in vitro. Infection of macrophage cultures with 15 PFU of Gilliam/cell resulted in 30% of the macrophages containing intracellular rickettsiae one day after infection (Fig. 2). There was a slight reduction in infected cells on day 2, followed by a constant increase throughout the remainder of the incubation period. This suggested rickettsiae were released

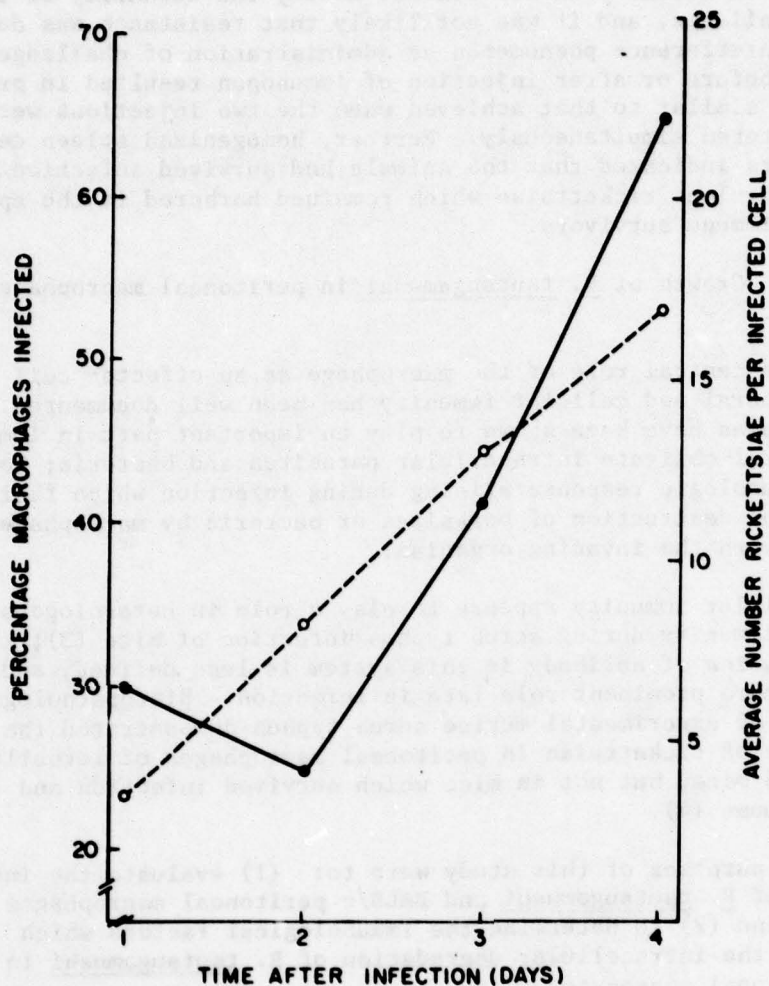


Figure 2. Growth of *R. tsutsugamushi*, strain Gilliam, in resident peritoneal macrophages. Symbols: (●) per cent macrophages infected, (○) average number of rickettsiae per infected cell.

from infected cells before macrophage lysis. Approximately 65% of the attached cells were infected with rickettsiae after 4 days of in vitro cultivation.

The average number of rickettsiae in each infected cell also increased with time, indicating rickettsial proliferation in macrophages. The initial exposure of macrophages to Gilliam resulted in 1-3 organisms per infected cell 24 hr after infection. The rickettsiae were localized in the perinuclear region of the cell, and the number of intracellular organisms progressively increased with continued incubation. By 5 days after infection, the entire cytoplasm of many of the macrophages was filled with rickettsiae.

The incorporation of radiolabeled adenine by irradiated L929 cell monolayers is enhanced during infection with *R. tsutsugamushi* (5). We employed a modification of this radioactive assay to determine rickettsial infection of macrophages. The technique complemented our microscopic studies and had the advantage of monitoring the entire cell population, both adherent and temporarily non-adherent macrophages. Figure 3 indicates that incorporation of ³H-adenine was significantly greater in Gilliam infected macrophages than in normal macrophages after 3 or 4 days incubation. These data directly supported our visual observations (Fig. 2) and established the capacity of scrub typhus rickettsiae to propagate in macrophages. We then examined several immunological factors which might enable the macrophage to control a rickettsial infection in vitro.

B. Effect of antibody on Gilliam proliferation in macrophages.

The optimum time and temperature of incubation of Gilliam with specific immune sera was determined to be 10 min at 37 C; this treatment optimized the effect of antibody while reducing the loss of rickettsial infectivity seen with longer extracellular incubation periods. The rickettsiae were treated with a final 1/2 dilution of immune or normal mouse serum and then added to peritoneal cell cultures for a standard adsorption period of 1 hr. In samples removed immediately after exposure to normal serum-treated rickettsiae, 20% of the macrophages contained an average of 1.2 intracellular organisms (Table 5). In contrast, treatment of rickettsiae with immune serum enhanced both the number of macrophages containing rickettsiae and the number of organisms per cell: 29% of these macrophages had 2.1 intracellular rickettsiae. Initial percentages of infected macrophages dropped from 30% for untreated rickettsiae (Fig. 2) to 20% for normal serum-treated

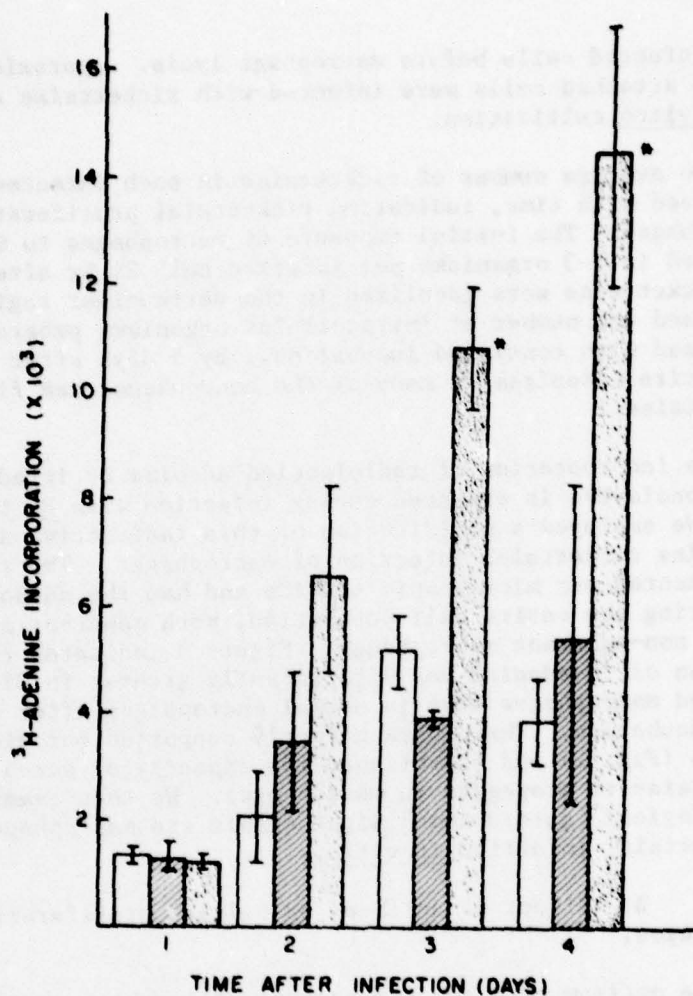


Figure 3. Incorporation of radiolabeled adenine by macrophage cultures. Open bars indicate uninfected macrophage cultures, shaded bars indicate macrophage cultures sham-inoculated with tissue culture material similar to that in which the rickettsial seed is grown, and stippled bars indicate rickettsiae-infected macrophage cultures. Significant difference from control cultures is denoted by * ($p < 0.05$).

Table 5. Infection of macrophages with R. tsutsugamushi strain Gilliam pre-treated with normal or immune mouse sera for 10 min at 37 C.

Time	% cells infected		No. rickettsiae per cell	
	Normal serum	immune serum	Normal serum	immune serum
0	20	29	1.2	2.1
1 day	18	8	3.1	3.5
2 days	17	7	8	7.4
3 days	22	9	14	13
4 days	50	32	>25	>25

rickettsiae (Table 5). This decrease was due to the extracellular incubation of rickettsiae rather than the normal mouse serum, since rickettsiae incubated in medium alone for 10 min at 37 C infected only 19% of the cells in a macrophage culture.

By day one post infection, the percentage of cells containing intracellular organisms in cultures exposed to immune serum-treated rickettsiae had decreased to 8%, indicating that antibody did enable a substantial number of macrophages containing intracellular rickettsiae initially to avoid infection. In contrast, cultures infected with normal serum-treated rickettsiae maintained an infection of 18%. The number of rickettsiae in infected macrophages of both cultures, however, increased at approximately the same rate over time, suggesting that the replication of rickettsiae in infected cells was not significantly affected by the presence of antibody. By the fourth day in culture, secondary infections were seen in macrophage cultures exposed to rickettsiae pretreated with both normal and immune serum: 50% of the macrophages exposed to normal serum-treated rickettsiae, and 32% of the macrophages exposed to immune serum-treated rickettsiae were infected. The majority of these cells contained rickettsiae in numbers too numerous to count.

Antibody clearly augmented the intracellular destruction of rickettsiae as evidenced by the two-fold reduction in the percentage of cells productively infected in cultures exposed to immune serum-treated rickettsiae compared to normal serum controls. The initial uptake and subsequent rickettsial proliferation in macrophage cultures infected with serum-treated rickettsiae was not enhanced further with the use of a high titered hyperimmune serum.

Antibody-coated rickettsiae are capable of penetrating and replicating in several cell types. This mode of entry into macrophages might circumvent the normal degradative process of phagolysosome formation and allow proliferation of the rickettsiae. A persistent fraction of the macrophages exposed to immune serum-treated rickettsiae did support the growth of scrub typhus, even in the presence of excess antibody. This suggested that additional mechanisms of enhancing rickettsial degradation by macrophages may be in effect during scrub typhus infection *in vivo*. This possibility was examined by treating macrophages with lymphokine-rich spleen cell supernatants prior to infection with rickettsiae.

C. Effect of lymphokine activation of macrophages on intracellular growth of Gilliam.

Spleen cells of BALB/c mice infected 21 days previously with Gilliam were cultured in the presence of heat-killed rickettsiae for approximately 30 hr. Culture supernatants of these cells

contained a lymphokine which activate normal macrophages for tumor cytotoxicity in vitro. The supernatants were applied to normal macrophage monolayers for 4 hr at 34 C; the cell sheets were washed and infected with viable rickettsiae. The results are shown in Fig. 4. By one day after infection, 19% of the macrophages exposed to control supernatants contained an average of 5 rickettsiae per infected cell. In contrast, only 4.5% of the macrophages exposed to the lymphokine-rich supernatants contained approximately 5 rickettsiae per cell. This 75% reduction in the number of infected macrophages in cultures treated with lymphokines persisted through 48 hr after infection. Rickettsiae did proliferate in infected cells of activated macrophage cultures; they reproduced more slowly in these cells, however than in untreated macrophages. These data suggest that the activated macrophage is capable of suppressing a rickettsial infection in vitro in the absence of specific antibody.

D. Effect of in vivo activation of macrophages on intracellular growth of Gilliam.

Suppression of rickettsial growth by macrophages activated in vitro could reflect an in vivo phenomenon during experimental scrub typhus infection. In order to investigate this possibility, macrophages were removed from Gilliam-infected mice at a time when the mice were immune to further challenge. Macrophages were harvested from mice inoculated i.p. 9 days previously with a non-lethal dose of Gilliam. These macrophages were shown by Giemsa-stained cytocentrifuge preparations to be free of intracellular rickettsiae and highly vacuolated at the time of harvest. Additionally, they were capable of killing tumor cells in the macrophage tumor cytotoxicity assay, an index of macrophage activation, suggesting that this was an activated macrophage population. When these activated macrophages were placed in culture and infected with approximately 15 PFU Gilliam/cell, only 5% of the macrophages contained intracellular rickettsiae one day after infection (Fig. 5). The number of infected macrophages actually decreased with time in culture. Comparison of these data with that of normal macrophage cultures (Fig. 2), where the 30% initially infected macrophages increased to 65% with 4 days incubation, suggested that macrophages activated during the course of scrub typhus infection suppressed rickettsial proliferation in vitro and prevented subsequent secondary infection of cells as well. The rickettsiae did increase in the small number of macrophages that sustained the rickettsial infection, as is seen by an increasing average number of intracellular rickettsiae over time (Fig. 5). Activated macrophages infected with Gilliam never incorporated more radiolabeled adenine than control cultures throughout the entire assay period (Fig. 6), indicating that the

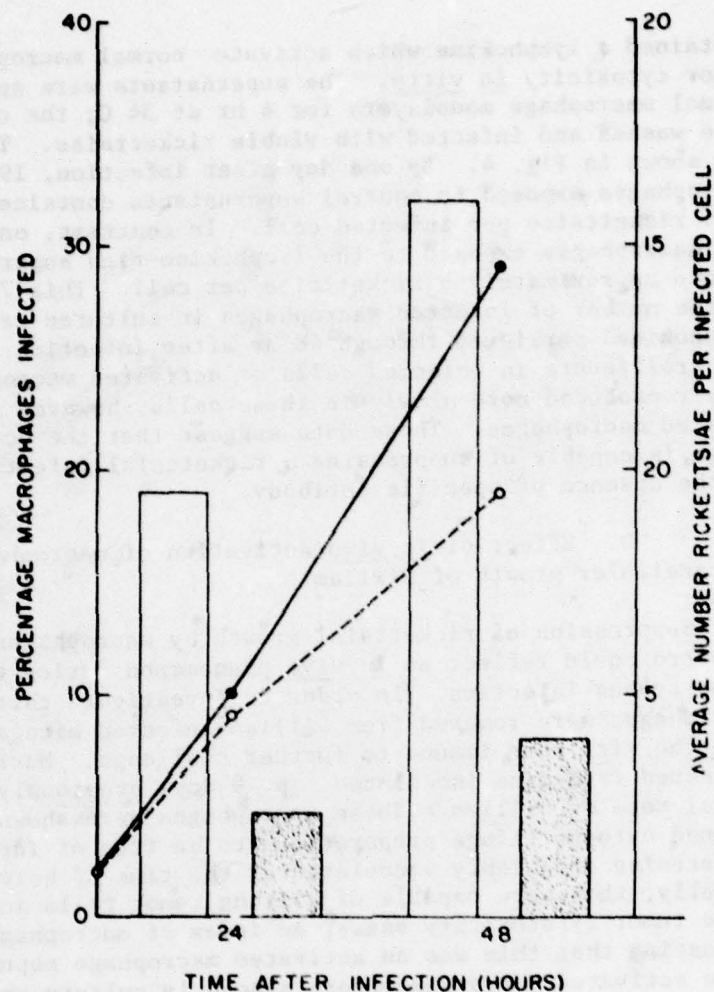


Figure 4. Effect of lymphokine activation of macrophages prior to rickettsial infection. Open bars indicate infected macrophages in cultures pretreated with spleen cell supernatants of normal mice, stippled bars indicate infected macrophages in cultures pretreated with lymphokine-containing spleen cell supernatants of Gilliam-infected mice. Symbols: (●) rickettsial growth in control macrophage cultures, (○) rickettsial growth in lymphokine-activated macrophage cultures.

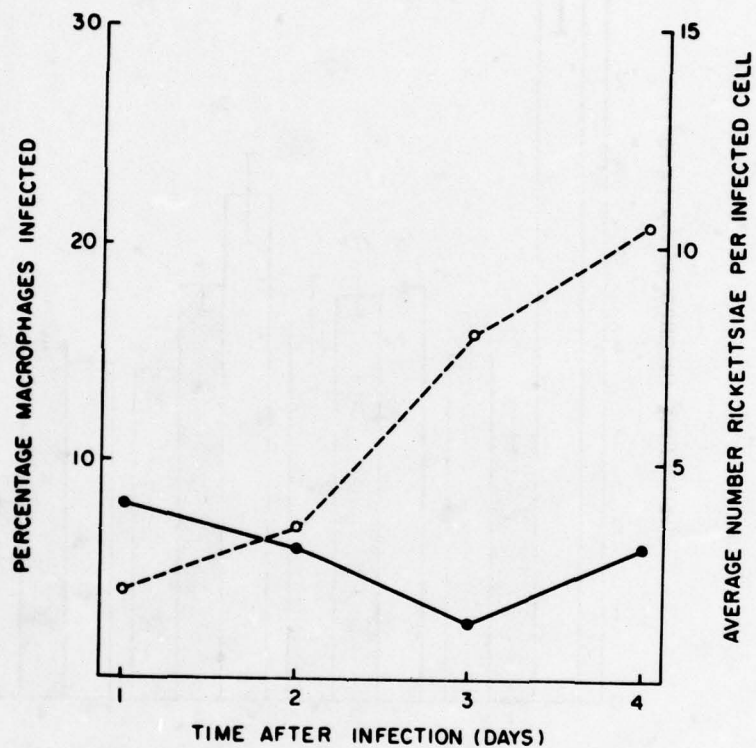


Figure 5. In vivo activation of macrophages prior to rickettsial infection: macrophages harvested from 9 day Gilliam-infected mice. Symbols: (●) percentage of macrophages infected, (○) average number of rickettsiae per infected cell.

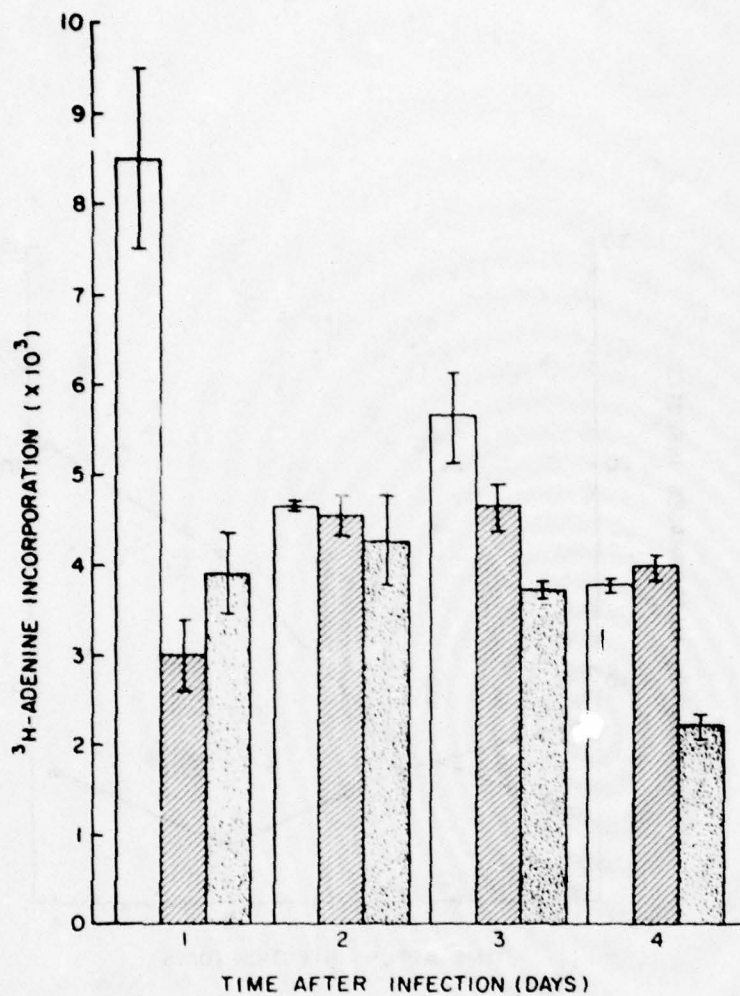


Figure 6. Incorporation of radiolabeled adenine by macrophage cultures activated *in vivo*: macrophages harvested from 9 day Gilliam-infected mice. Open bars indicate uninfected macrophage cultures, shaded bars, macrophages sham-infected with tissue culture material similar to that in which rickettsial seed is grown, and stippled bars, macrophages exposed to rickettsiae.

decrease in infected macrophages observed microscopically was not due to cell detachment. These data suggest that macrophages activated in vivo are also refractory to rickettsial infection in vitro.

Both the visual and radioactive data support the conclusion that normal macrophages do not control rickettsial infection once the rickettsiae are established in the cytoplasm of the cell. The rickettsiae appeared to multiply in macrophages at about the same rate they replicate in non-professional phagocytic cells. The number of infected macrophages, as well as the number of intracellular rickettsiae, increased with continued incubation, suggesting that the rickettsiae were indeed replicating in the infected cells and were capable of migrating from infected macrophages prior to cell lysis. The incorporation of radiolabeled adenine into infected macrophage cultures clearly supported the data obtained by visual examination of the cells. Although the sensitivity of the assay was not sufficient to distinguish the initial infection, subsequent rickettsial proliferation and secondary infection were easily discernable. Infected macrophages incorporated significantly greater adenine than control cultures by 3 and 4 days of incubation, a time at which approximately 40% of the macrophages contained greater than 10 rickettsiae per cell.

In this study, we investigated the ability of antibody and lymphokines to enable normal macrophages to suppress rickettsial growth in vitro. The sequence of events leading to active immunity in rickettsial infections is, at best, incompletely understood. Since the macrophage plays such a crucial role in both the afferent and efferent aspects of an immunological response, they are probably rendered capable of suppressing rickettsial growth early in infection. In other bacterial systems, elimination of bacteria by macrophages is facilitated by the presence of specific antibodies or macrophage-activating lymphocyte products.

Because macrophages have surface receptors for the Fc portion of immunoglobulins, a humoral immune response greatly augments macrophage phagocytosis and disposal of antigens. Treatment of R. tsutsugamushi with immune serum prior to infection of resident peritoneal macrophages enhanced the initial uptake of rickettsiae by these cells. Comparison of the number of macrophages containing intracellular organisms immediately and one day after exposure to immune serum-treated rickettsiae suggested that many of the treated rickettsiae entering the macrophages were subsequently digested by the cells. Overall, comparing macrophage cultures exposed to immune serum- and normal serum-treated rickettsiae, there was a two-fold reduction in the number of infected macrophages when the rickettsiae were pretreated with antibody. The

effect of antibody on scrub typhus infection, however, was not as dramatic as reported for R. prowazeki in human macrophages, where virtually 100% of the cells were refractory to rickettsial infection in the presence of antibody.

A persistent fraction of macrophages exposed to immune serum-treated scrub typhus rickettsiae became infected and supported the growth of rickettsiae. Earlier studies have suggested that antibody coated R. tsutsugamushi is fully capable of penetrating and replicating in non-phagocytic cells. Rickettsial penetration of the macrophage, circumventing the normal degradative process of phagolysosome formation, may account for the persistent fraction of infected macrophages following exposure to immune serum-treated rickettsiae. Another possibility is that the population heterogeneity suggested by the low initial susceptibility of normal macrophages is a functional heterogeneity. R. tsutsugamushi was capable of infecting in culture approximately 30% of the adherent resident peritoneal cells of BALB/c mice. This initial infectivity of rickettsiae for macrophages was the same over a multiplicity of infection (MOI) of 3-30 PFU per cell and may reflect a remarkably consistent heterogeneous susceptibility of the cell population. A standard MOI of 15 PFU per macrophage was used throughout the study. Cells that become infected following exposure to immune serum-treated rickettsiae may not have Fc receptors, may not be as highly phagocytic as cells resisting infection, or may normally respond to lymphocyte products in a cellular response.

Macrophages activated by lymphokines in vitro are capable of suppressing rickettsial growth in the absence of antibody. When normal macrophages were subjected to lymphokines containing no detectable rickettsial antibody, there was a 75% reduction in the number of infected macrophages one day after exposure to rickettsiae. This reduction was constant through 48 hr. Rickettsial proliferation within infected cells of the activated macrophage cultures was slower (two-fold increase in intracellular rickettsiae) than in control macrophage cultures (three-fold increase). The activated state of macrophages exposed to lymphokines in vitro is relatively short; therefore, the lymphokine experiments were terminated prior to the time secondary infections occur in normal macrophage cultures.

Cellular immunity appears to play an important role in resistance to rickettsial disease. This is suggested by the appearance of delayed hypersensitivity (6) and the demonstration of several correlates of cellular immunity in vitro following recovery from infection (7). In experimental scrub typhus infection of mice, heterologous strain immunity was adoptively transferred with splenic

T cells from inoculated mice as early as 7 days after infection (3). Since normal resident macrophages exposed to lymphokines in vitro were capable of suppressing rickettsial growth, we were interested in determining whether macrophages become activated for rickettsial suppression in the early stages of rickettsial disease, when cellular immunity is present but circulating antibody cannot be demonstrated. Peritoneal macrophages from mice inoculated 9 days previously with Gilliam and free of intracellular rickettsiae were capable of killing tumor cells in vitro, an index of macrophage activation. They were also capable of suppressing rickettsial infection, since only 5% of the cells contained intracellular organisms one day after exposure to viable rickettsiae. In contrast to in vitro lymphokine stimulation, the number of infected macrophages in this population actually declined with time in culture, and secondary infections were not evident with either visual or radioactive data. Macrophages activated during the course of rickettsial infection in vivo, then, are also capable of suppressing rickettsial growth.

Clearly, there are several ways in which a resident peritoneal macrophage may become resistant to scrub typhus infection. Neither antibody nor lymphokine-activation alone was capable of reducing rickettsial infection of mouse macrophages completely. This suggests a cooperative response between cellular and humoral immunity in the intact animal may be necessary to completely resist infection. Our findings do, however, extend our previous observations on the role of cellular immunity in primary scrub typhus infections (3), as generation of lymphokine-activated macrophages both in vivo and in vitro renders the cells refractory to rickettsial infection. Further studies on the mechanism(s) by which activated macrophages suppress rickettsial infection are currently in progress.

III. Mapping of the gene controlling natural resistance to R. tsutsugamushi infection in mice.

Inbred mouse strains differ dramatically in their natural resistance to a number of infectious agents. Resistance to certain viral agents has been linked to the H-2 complex and may represent the effect of Ir gene function. However, susceptibility to a variety of bacterial, viral, and protozoal infections has been found to be under non-H-2 linked genetic control which may reflect resistance mechanisms other than those involving specific immune recognition. We recently reported that natural resistance to the Gilliam strain of R. tsutsugamushi was controlled by a single, autosomal, dominant, non-H-2 linked gene.

In the present investigation, we have continued these studies and attempted to map the chromosomal location of this gene. Using recombinant inbred (RI) strains of mice, we have found that this gene is located on chromosome 5, closely linked to the retinal degeneration (rd) locus, and have confirmed this with a backcross linkage analysis. We have assigned to it the designation Ric, with r and s representing the resistant and susceptible alleles respectively.

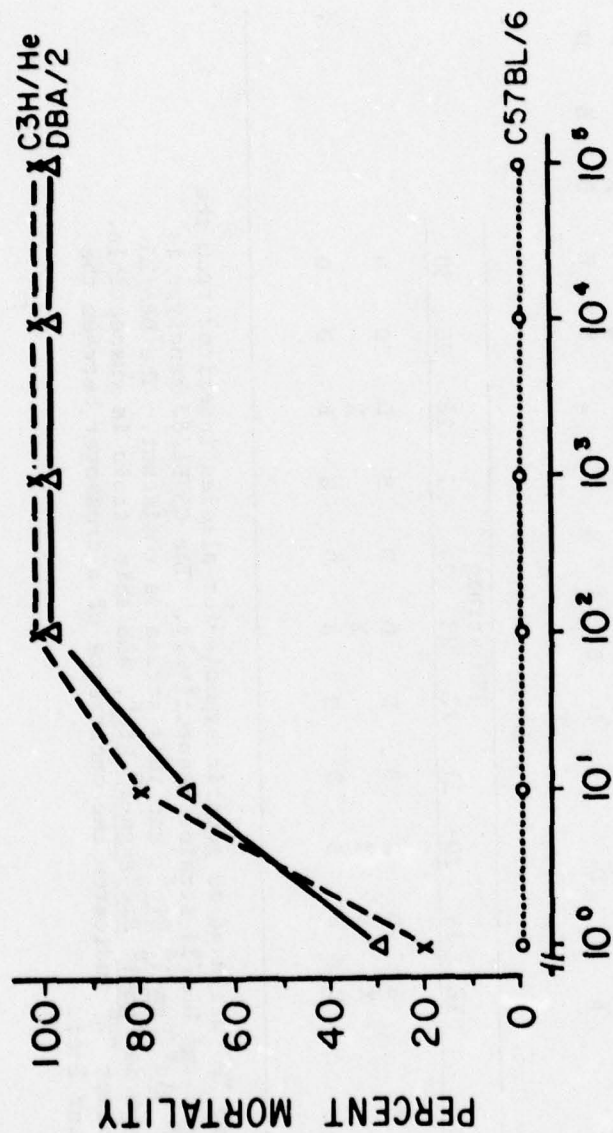
A. Response of inbred mouse strains to R. tsutsugamushi.

Three strains, C57BL/6J, C3H/HeJ and DBA/2J were chosen for analysis because they were either resistant or susceptible and the progenitors of two large sets of RI mouse strains. The relative resistance of these strains to R. tsutsugamushi Gilliam strain is illustrated in Fig. 7. C57BL/6J mice were resistant to all doses of R. tsutsugamushi tested up to a dose of 10^5 MID₅₀. In contrast, 100% of C3H/HeJ and DBA/2J mice were killed by 10^2 MID₅₀. From the figure, it is clear that a dose of 10^3 MID₅₀ will kill all susceptible mice and not kill resistant mice. Therefore, this dose was utilized for the remainder of the study.

B. Response of recombinant inbred (RI) mouse strains to R. tsutsugamushi.

Two RI lines were used to map the rickettsial gene. RI strains are powerful tools for the study of genetic segregation, linkage, and pleiotropic effects of allelic differences (9). Such strains are produced by inbreeding, beginning with randomly chosen pairs of mice from the F₂ generation of the cross between two dissimilar progenitor strains. In our study, the BXD RI strains were derived from a cross of C57BL/6J and DBA/2J mice, and the BXH strains were derived from a cross of C57BL/6J and C3H/HeJ. Twenty-three different BXD strains (3 mice/strain) were infected with 10^3 MID₅₀ of R. tsutsugamushi, and their susceptibility determined. Only groups showing 100% survival or resistance in an individual trial were included. Eleven strains were resistant and 12 susceptible (Table 6). Of all the known mapped chromosomal markers in these lines, analysis of the pattern of resistance suggested a possible linkage to the chromosome 5 marker for phosphoglucomutase-1 (Pgm-1), since only 8 of 23 strains reveals crossovers between Ric and Pgm-1. Had no linkage occurred, approximately 50% of the strains would have exhibited crossovers.

In order to confirm this linkage we next analyzed the resistance pattern of 13 BXH strains, which have been typed for 2 additional chromosome 5 markers, glucuronidase (Gus) and retinal degeneration (rd). Nine strains were susceptible and 4 resistant



MOUSE ID₅₀ GILLIAM

Figure 7. Susceptibility of inbred mouse strains used in recombinant and backcross studies to the Gilliam strain of R. tsutsugamushi.

Table 6. Susceptibility of BXD recombinant inbred mice to Rickettsia tsutsugamushi.

Locus	C57BL/6J	DBA/2J	BXD strain													
			1	2	5	6	8	9	11	12	13	14	15	16		
<u>Pgm-1</u>	B	D	B	D	D	B	B	D	B	B	B	B	B	B	D	
<u>Ric</u>	B	D	B	D	D	B	B	B	D	D	B	D	B	D	D	

Locus	18	19	20	21	22	23	24	27	28	28	30		
<u>Pgm-1</u>	B	D	D	D	D	D	D	B	D	D	D		
<u>Ric</u>	X	X	X		X				X				
	D	B	B	D	D	B	D	B	B	D	D		

"B" and "D" are used as generic symbols for alleles inherited from the C57BL/6J and DBA/2J strains respectively. The C57BL/6J genotype is Pgm-1^a Ric^r/Pgm-1^a Ric^r, and this strain is resistant. The DBA/2J genotype is Pgm-1^b Ric^s/Pgm-1^b Ric^s, and this strain is susceptible. The letter "X" indicates the occurrence of a crossover between the adjacent loci.

to R. tsutsugamushi (Table 7). Analysis of the pattern of resistance in these strains confirmed the linkage to Pgm-1 and, in addition, suggested that Ric was closely linked to rd since there was no crossover between Ric and rd. In contrast, there were 4 crossovers between Pgm-1 and Ric and 5 crossovers between Gus and Ric.

C. Backcross linkage analysis between Ric and chromosome 5 markers.

We next performed a backcross linkage analysis in order to obtain a better estimate of the distance between rd and Ric and to possibly establish the correct gene order for these two loci. (C57BL/6J x DBA/2J) F₁ male mice were backcrossed to DBA/2J females. The progeny were first typed for Pgm-1 and subsequently tested for resistance to R. tsutsugamushi. Of the 53 mice tested, there were only 10 recombinants (Table 8). Six resistant mice possessed the Pgm-1 genotype of the susceptible DBA/2J parent (bb), while 4 susceptible mice possessed the genotype of the F₁ resistant parent (ab). These results confirmed the linkage of Ric to Pgm-1.

In order to analyze the distance of Ric from rd, (C57BL/6J x C3H/HeJ) F₁ mice were backcrossed to C3H/HeJ females. The progeny were first tested for rd and, subsequently, tested for rickettsial resistance. Only three crossovers between Ric and rd were found among the 50 backcross progeny tested (Table 9).

In order to determine the gene order, a three-point cross was performed. Twenty-five of the backcross progeny listed in Table 9 were tested for Pgm-1, rd, and Ric. Only two crossovers between Ric and rd were found out of the 25 backcross mice tested (Table 10). In these two mice, the Pgm-1 genotype remained linked to the rd genotype, and no double crossovers occurred suggesting the gene order Pgm-1-rd-Ric.

The data presented here demonstrate that Ric, the gene controlling resistance to scrub typhus in mice, is located on chromosome 5 closely linked to the gene that controls retinal degeneration (rd). No crossovers between Ric and rd were found among the 13 BXH RI strains tested and only 3 recombinants between Ric and rd occurred among 50 backcross progeny.

We propose the gene order Pgm-1-rd-Ric-Gus since in the two crossovers found in the backcross progeny, the Pgm-1 and rd genotypes remained linked. Interchanging rd and Ric would require postulating multiple crossovers.

Table 7. Susceptibility of BXH recombinant inbred mice to Rickettsia tsutsugamushi.

Locus	C57BL/6J	C3H/HeJ	BXH strain																
			2	3	4	5	6	7	8	9	10	11	12	14	19				
<u>Pgm-1</u>	B	H	H	H	H	B	B	B	H	H	H	H	B	H	H				
<u>rd</u>	B	H	H	H	H	X	H	X	H	H	B	B	B	B	H				
<u>Ric</u>	B	H	H	H	H	H	B	H	H	H	B	B	E	H	H				
<u>Gus</u>	B	H	X	H	X	X	B	X	H	H	X	B	B	X	B				

"B" and "H" are used as generic symbols for alleles inherited from the C57BL/6J and C3H/HeJ strains, respectively. The C57BL/6J genotype is Pgm-1^a Ric^r rd⁺ Gus^b / Pgm-1^b Ric^s rd^o Gus^h, and this strain is resistant. The C3H/HeJ genotype is Pgm-1^b Ric^s rd^o Gus^h / Pgm-1^b Ric^s rd^o Gus^h, and this strain is susceptible. The letter "X" indicates the occurrence of a crossover between the adjacent loci.

Table 8. Summary of backcross linkage analysis between Pgm-1 and Ric.

<u>Pgm-1</u> genotype	No. of resistant mice (<u>Ric</u> ^r / <u>Ric</u> ^s)	No. of susceptible mice (<u>Ric</u> ^s / <u>Ric</u> ^s)
ab (F ₁ type)	28	4
bb (DBA/2 type)	6	15

(C57BL/6J x DBA/2J) F₁ mice were backcrossed to DBA/2J females and the progeny were typed for Pgm-1 and Ric.

Table 9. Summary of backcross linkage analysis between Ric and rd.

<u>rd</u> genotype	No. of resistant mice (<u>Ric</u> ^r / <u>Ric</u> ^s)	No. of susceptible mice (<u>Ric</u> ^s / <u>Ric</u> ^s)
<u>rd</u> /+ (F ₁ type)	24	3
<u>rd</u> / <u>rd</u> (C3H/He type)	0	23

(C57BL/6J x C3H/HeJ) F₁ mice were backcrosses to C3H/HeJ females and the progeny were tested for rd and Ric.

Table 10. Segregation of Pgm-1, rd and Ric markers in backcross C3H/HeJ X (C57BL/6J X C3H/HeJ) F₁ mice.

Region of recombination	Genetic locus			No. of mice
	<u>Pgm-1</u>	<u>rd</u>	<u>Ric</u>	
None	<u>a</u>	+	<u>r</u>	8
	<u>b</u>	<u>rd</u>	<u>s</u>	10
<u>Pgm-1-rd</u>	<u>a</u> X	<u>rd</u>	<u>s</u>	3
	<u>b</u> X	+	<u>r</u>	2
<u>rd-Ric</u>	<u>a</u>	+	<u>s</u>	2
	<u>b</u>	<u>rd</u> X	<u>r</u>	0
<u>Pgm-1-rd-Ric</u>	<u>a</u> X	<u>rd</u> X	<u>r</u>	0
	<u>b</u> X	+	<u>s</u>	0
Total				25

The parental genotypes for the Pgm-1, rd, and Ric markers respectively are:

C3H/HeJ: b rd s/b rd s

C57BL/6J: a + r/a + r

The recombination frequency (RF) between rd and Ric was .06 (3/50) in the backcross progeny. No recombinants between rd and Ric were found among 13 RI strains. Thus, we can calculate the RF between these loci to be .06 or less.

The recombinates between Pgm-1 and Ric in the RI strains (BXD and BXH) was 14/36. Using available formulae the RF can be estimated to be 0.23. This estimate is an excellent agreement with that obtained from the backcross data 0.22 (17/78). This distance is also consistent with the gene order Pgm-1-rd-Ric because interchanging these loci would place Ric much closer to Pgm-1.

As far as we can determine, there is no connection between Ric and the genes that control natural resistance to any other tested infectious organism. Bradley et al. have recently mapped a gene that controls resistance to Leishmania donovani (Lsh) in mice to chromosome 1 (Mouse Newsletter, July 1978, pg. 56-57). Therefore, Lsh is clearly different from Ric. Similarly, comparison of the distribution of resistance patterns among inbred mouse strains to Salmonella typhimurium, Listeria monocytogenes, Corynebacterium kutscheri, Toxoplasma gondii, or Herpes simplex virus indicates that Ric does not control resistance to any of these agents. Furthermore, the locus does not even control resistance to every strain of R. tsutsugamushi because all strains of mice tested have been equally susceptible to the Karp strain of R. tsutsugamushi (8).

The gene product of the Ric locus or the mechanism of its action remains unknown for the present. The only gene that maps close to Ric that may be involved in resistance is a minor histocompatibility locus, H(go). Interestingly, one of the genes that controls resistance to T. gondii has been linked to another minor histocompatibility locus, H-13. Therefore, this connection may be worth pursuing.

The Ric locus does not control the growth of R. tsutsugamushi in cells because these organisms grow equally well in fibroblasts derived from resistant or susceptible mice (8). In addition, there is no apparent defect in immune recognition in susceptible mice because they can be protected against subsequent i.p. infection by a subcutaneous inoculations of a virulent R. tsutsugamushi (8). This finding also demonstrates that susceptible mice possess the appropriate cellular machinery (i.e. macrophages, granulocytes and immunoglobulin) to control a rickettsial infection. The only reported difference between susceptible and resistant strains is that peritoneal macrophages of susceptible mice are killed during in vivo, R. tsutsugamushi infection while those of resistant mice survive.

However, two other linkages do suggest possible mechanisms of action of the Ric locus. The chronic granulomatous reaction of mice to killed BCG organisms was controlled by a single autosomal dominant gene. Interestingly, the mouse strain distribution of responsiveness to this BCG effect correlated with that of Ric. A similar correlation has been found in the ability of mice to mount an acute inflammatory response. Therefore, it is possible that the Ric locus controls the ability of mice to mount a vigorous acute and/or chronic inflammatory responses which would serve to control a rickettsial infection in a non-antigen specific manner.

The elucidation of the product of the Ric gene will require extensive investigation. Nonetheless, knowing the chromosomal location of this locus, in addition to the availability of a readily accessible closely linked marker (rd) should greatly facilitate these studies.

IV. Electron microscope study of mouse capillary endothelium infected with R. tsutsugamushi.

Rickettsia tsutsugamushi, the etiologic agent of scrub typhus, is an obligate intracellular parasite transmitted by the bite of a larval mite. Rickettsiae enter the bloodstream and infect capillary endothelial cells throughout the body. Yet little is known about the morphologic character of the resultant vascular lesions. We have found the capillary endothelium of BALB/c mouse cerebellum to be suitable for study of these lesions by EM. Mice were sacrificed 6 days after intravenous inoculation of 1.2×10^6 50% mouse lethal doses of egg-grown R. tsutsugamushi, strain Karp.

Rickettsiae were identified free within endothelial cytoplasm. Compared to controls, infected endothelial cells were swollen and contained increased numbers of cytoplasmic components including mitochondria and lysosomes (Fig. 8-11). Occasional giant mitochondria were found in infected cells (Fig. 12). There were focal perivascular hemorrhages and intravascular fibrin thrombi (Fig. 13). However, the junctional complex and basal lamina of endothelium and pericytes remained unchanged (Fig. 14). Rickettsiae were released from endothelial cells into the capillary lumen by budding through the host cell plasma membrane (Fig. 15).

These results suggest that capillary endothelial cells respond to rickettsial infection with hypertrophy rather than degeneration and their cytoplasmic changes represent a morphologic expression of altered cellular metabolism of infected cells.

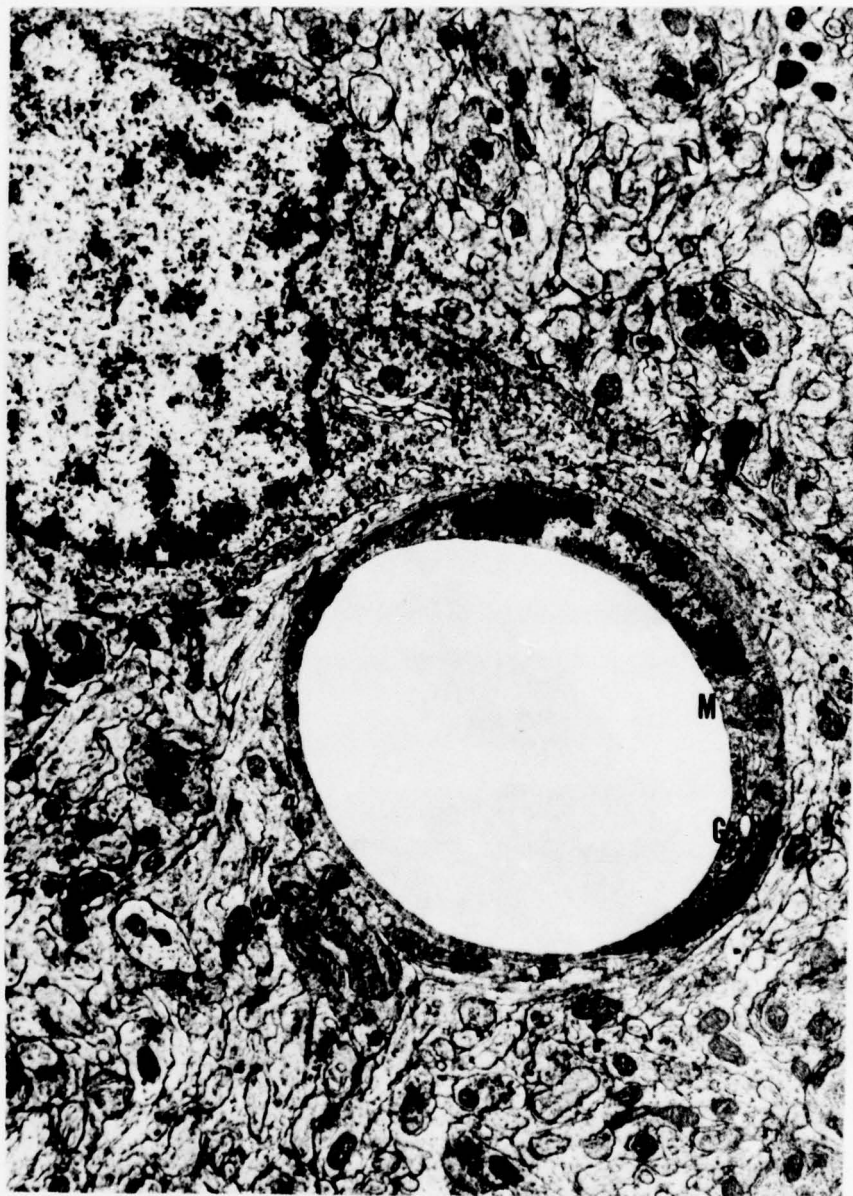


Figure 8. CONTROL - The endothelial cytoplasm contains a few mitochondria (M) and Golgi lamellae (G). There is no perivascular edema.



Figure 9. HEMORRHAGE - Several red blood cells (RBC) have broken out of a capillary, while others adhere to the capillary luminal surface. There is marked perivascular edema. The endothelium is necrotic and shows several rickettsiae (R), a pyknotic nucleus (N) and swollen mitochondria (M). Several oligodendrocytes (Ol) are visible.



Figure 10. MACROPHAGE - Prowling the endothelial surface of this capillary cut in longitudinal section is one of many macrophages observed in infected cerebellar capillary beds. The macrophage is itself infected with several rickettsiae (R).



Figure 11. HYPERTROPHY - Endothelial cell infected with rickettsiae (R) is thickened and contains abundant mitochondria (M) and Golgi lamellae (G). The clear space around the organisms is probably an artifact.



Figure 12. GIANT MITOCHONDRION - Endothelial cells sometimes contained not only greater than normal numbers of mitochondria, but occasional giant mitochondria (M). Secondary lysosomes (L) were often present.



Figure 13. THROMBUS - A mass of fibrin and cell debris projects into a capillary lumen (C) from a denuded area of the basal lamina (arrow), flanked by endothelial cells containing a rickettsia (R) and an occasional secondary lysosome (L).



Figure 14. INTACT JUNCTION - With rare exceptions, endothelial cell-to-cell junctions were maintained even when the cells were heavily infected. A hypertrophied endothelial cell containing a rickettsia (R) remains firmly attached to an adjacent endothelial cell.



Figure 15. BUDDING - Partially enclosed by host cell plasma membrane, a rickettsia (R) is being released from the luminal surface of a capillary endothelial cell. This host membrane coat is retained by the organism following its release.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 006 Rickettsial Diseases of Military Personnel

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1. Nacy-Mahady, C., Metzer, M., Osterman, J.V., and Brandt, W. Suppression of rickettsial growth in macrophages activated by antigen-stimulated spleen cell supernatants. ASBC/AAI Annual Meeting, Atlanta, Georgia, 1978 (June).
2. Nacy-Mahady, C. and Osterman, J.V. Growth of Rickettsia tsutsugamushi in peritoneal macrophages of mice. Annual Meeting of the American Society for Microbiology, Las Vegas, Nevada, 1978 (May).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL
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ADDRESS: Washington DC 20012				ADDRESS: Kuala Lumpur, Malaysia		
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)		
NAME: RAPMUND, Garrison, COL, MC				NAME: Huxsoll, D.L., LTC, VC		
TELEPHONE: 202-576-3351				TELEPHONE: 984155, 984249		
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30. TECHNICAL OBJECTIVE, 31. APPROACH, 32. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)						
<p>23(U) To investigate the incidence of scrub typhus infection in selected local and foreign populations, to relate this to the prevalence of vector chiggers and their common rodent hosts, to determine the strains of R. tsutsugamushi causing human infections and relate to clinical disease, to develop improved serological methods, to evaluate simple and effective means of treatment, to study the basis for immunity, to define vector-rickettsia relationships, to develop a laboratory animal model for the disease, and to produce laboratory animals in support of the mission. Scrub typhus is an incapacitating disease of military importance.</p> <p>24(U) Provides scientific reagents and equipment which can be best procured in the US for shipment to and use by WRAIR personnel in Malaysia functioning collaboratively with Malaysian scientists supported under USAMRDC grant reported on DA OA 7413.</p> <p>25(U) 77 10 - 78 09 Studies have shown: a correlation between the high incidence of scrub typhus among settlers on new oil palm schemes and a high density of vector chiggers; a majority of confirmed scrub typhus cases become serologically negative in the IFA test within 18 months following infection; Leptotrombidium viverricola may be an important vector of scrub typhus in Malaysia; Rickettsia tsutsugamushi can be isolated in monocyte cell cultures derived from experimentally infected monkeys and dogs; R. tsutsugamushi isolated from infected laboratory reared L. fletcheri chiggers consists of both Karp and Gilliam strains; young cynomolgus monkeys may prove to be a useful laboratory animal for scrub typhus studies; dogs experimentally infected with Ehrlichia canis and treated with prophylactic tetracycline for 30 days are cleared of the infection. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.</p>						

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 007 Field Studies of Rickettsioses and Other Tropical Diseases

Investigators:

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THE LONGEVITY OF ANTIBODY TO RICKETTSIA TSUTSUGAMUSHI IN PATIENTS
WITH CONFIRMED SCRUB TYPHUS

Background: Previous estimates of the longevity of antibody are limited to those of Bozeman and Elisberg (3) who studied the antibody response by indirect immunofluorescence in volunteers and laboratory personnel known to have been infected with R. tsutsugamushi. These workers showed that persistence of antibody in two individuals following primary infection differed; however, in both individuals they found significant titers 12 years after reinfection. Recent studies in this laboratory suggest that antibody may disappear rapidly following symptomatic infections. Animal studies at this unit (43) also suggest antibody persistence of less than 1 year after primary infection in silvered leaf monkeys (Presbytis cristatus). No other publication has specified the rate of decay of human antibody or the factors affecting it, yet such information is crucial to the interpretation of antibody prevalence surveys (44).

Materials and Methods: The population investigated was from those patients presenting to a field study site at a rural Malaysian hospital or another at a FELDA oil palm scheme in Western Pahang with a confirmed diagnosis of scrub typhus. Diagnosis was determined by one or more of the following criteria in association with an otherwise unexplained febrile illness (5):

- (i) isolation of the organism
- (ii) a four-fold rise in IFA titer to at least 1:200
- (iii) a four-fold rise in Proteus OXK agglutinin titer to at least 1:160

Further selection occurred by other uncontrolled factors such as the geographic accessibility of the subject. All patients came from areas of high endemicity.

Serial blood samples from 114 patients were collected at varying intervals during and for up to two years after the initial disease episode. Serum was separated from the collected blood, frozen and stored at -20°C until tested in the laboratory. Samples were subsequently tested for antibodies to R. tsutsugamushi by the indirect fluorescent antibody test (IFAT) (41). The test was slightly modified to test sera against only two antigens: the first was polyvalent and included the Karp, TA716 and TA763 strains, and the second, Gilliam strain alone. The reason for this modification was the fact that irrespective of whether or not R. tsutsugamushi was isolated and irrespective of the antigenic character of any isolate, antibodies to all strains appeared. Gilliam was tested separately as antibody to it was thought to have a different pattern of persistence.

Peak titer was defined as the highest titer reached in the samples obtained. When more than one sample from a single subject had the same peak titer, it was timed as the last sample in the initial four-week period from onset of illness or, if all such samples occurred outside that initial period, as the first such sample.

No result after the peak titer was excluded from analysis unless the criteria for re-infection were met. These criteria were the same as for the initial diagnosis of scrub typhus.

Analysis was by multiple linear regression of three variables (34) - peak titer, surveillance titer, and, as dependent variable, elapsed time from point of peak titer to survey point. All variables were expressed as exponents.

Results: The peak titers of the 114 patients for the two antigens were distributed as follows:

Karp + TA716 + TA763 (n = 114):

Level: Mean exponent 4.32 (titer 1:499)

s 1.37, SE 0.13 (Figure 1)

Timing: Mean \log_{10} time 0.44 (2.75 weeks)

s 0.27, SE 0.03 (Figure 2)

Gilliam (n = 112):

Level: Mean exponent 4.18 (titer 1:453)

s 1.80, SE 0.17 (Figure 3)

Timing: Mean \log_{10} time 0.44 (2.75 weeks)

s 0.26, SE 0.02 (Figure 4)

In all cases, multiple regression analysis gave a highly significant ($P < 0.0005$) correlation and explained approximately 10% more of the variation than did the simple regression of fall in titer on time.

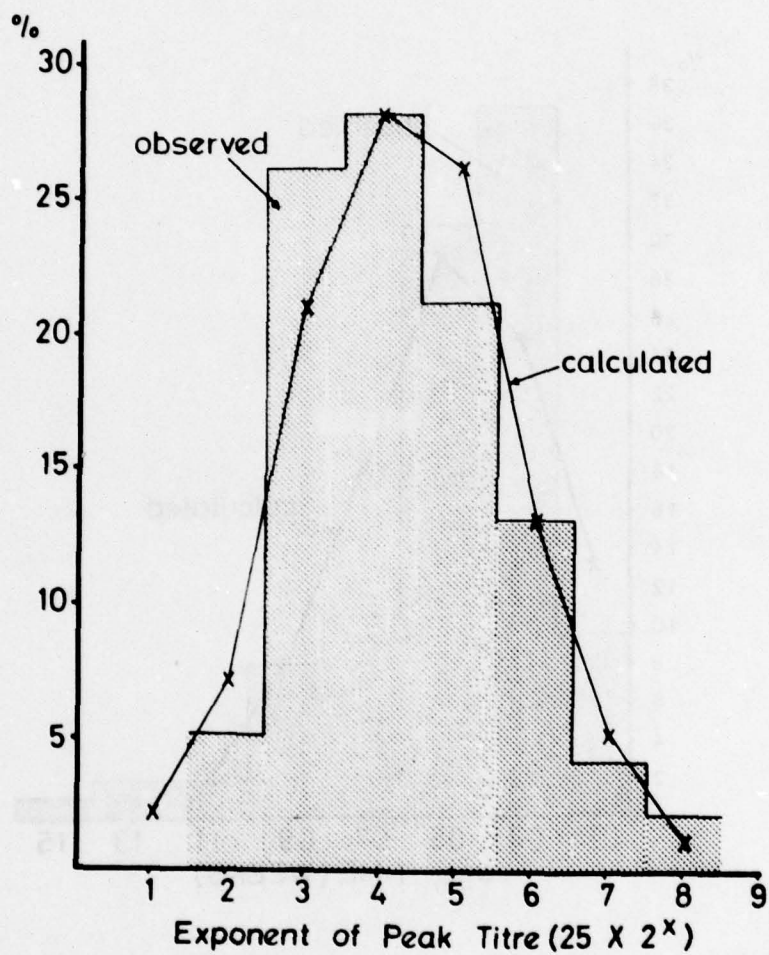


Figure 1. Distribution of peak titer of antibody to Karp/716/763 strains of *R. tsutsugamushi* showing observed and calculated frequencies.

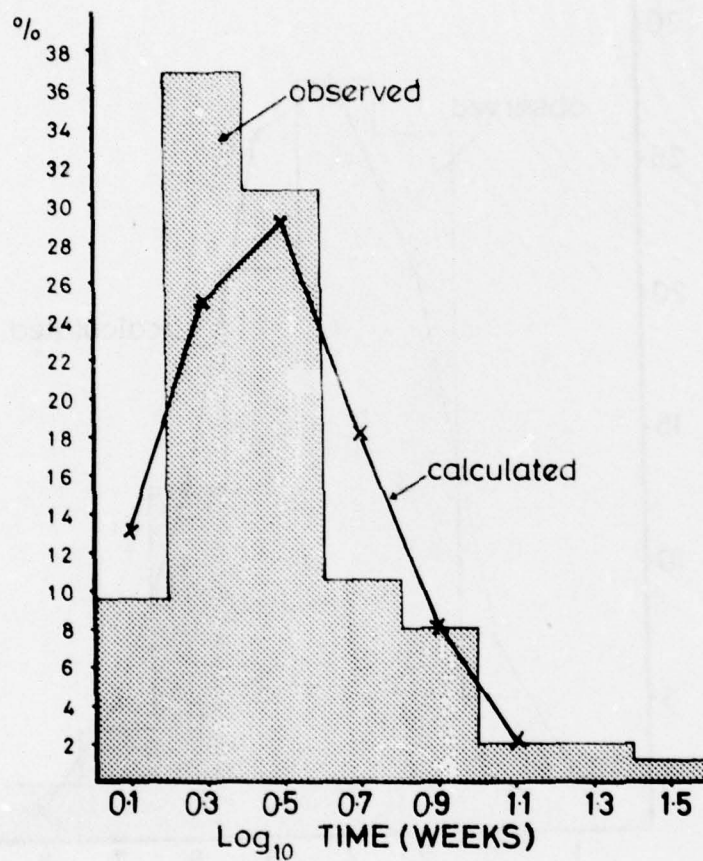


Figure 2. Distribution of time of occurrence (with respect to claimed start of illness) of peak titer of antibody to Karp/716/763 strains of *R. tsutsugamushi* showing observed and calculated frequencies.

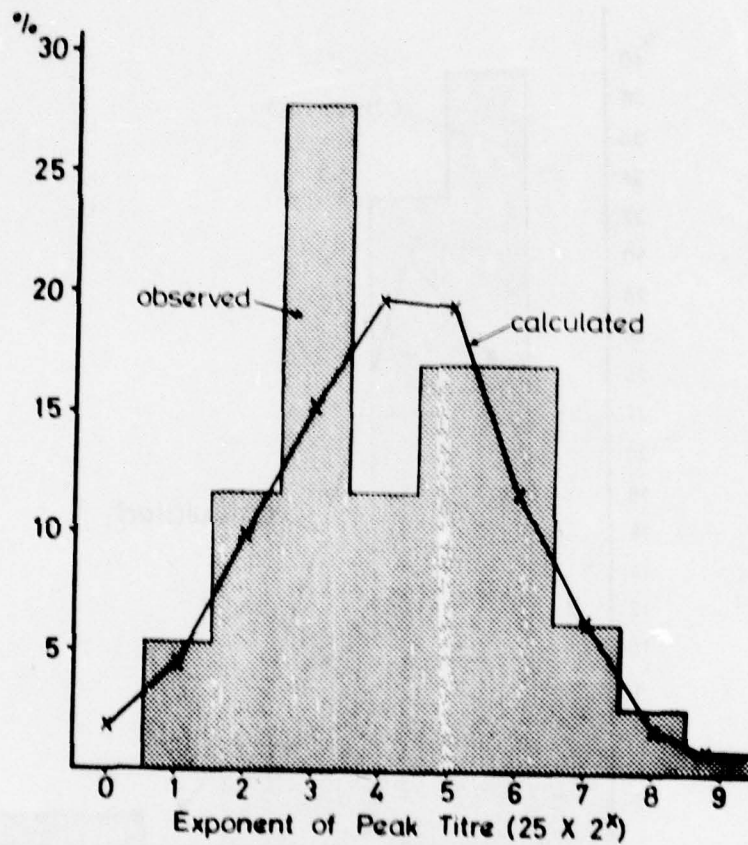


Figure 3. Distribution of peak titer of antibody to Gilliam strain of *R. tsutsugamushi* showing observed and calculated frequencies.

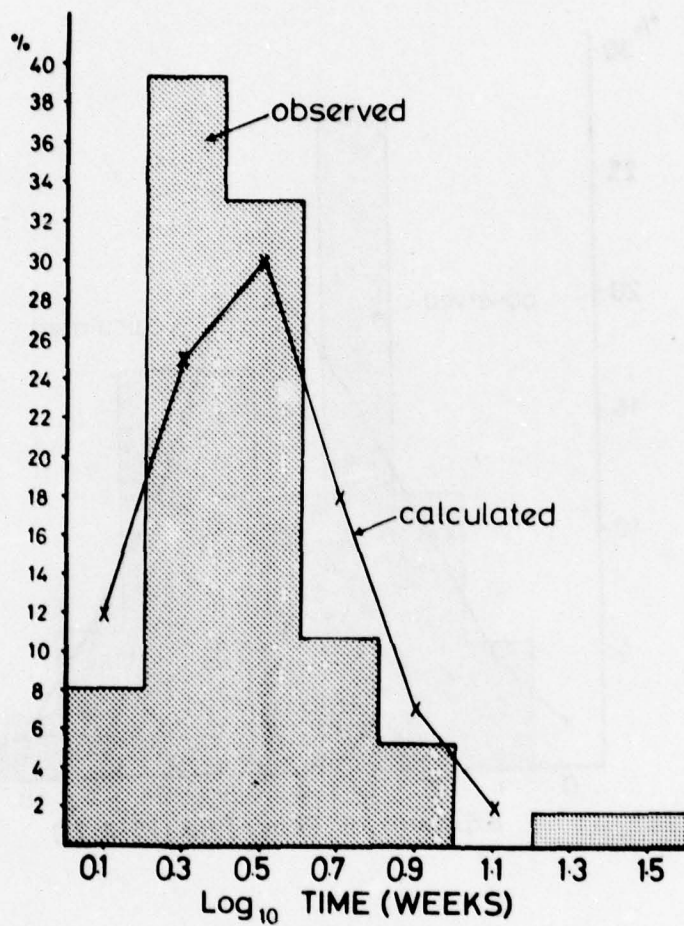


Figure 4. Distribution of time of occurrence (with respect to claimed start of illness) of peak titer of antibody to Gilliam strain *R. tsutsugamushi* showing observed and calculated frequencies.

For antibody to the polyvalent antigen (Karp, TA763, TA716) the calculated regression is:

$$\text{Log}_{10} T = 1.43 + 0.06 D_p - 0.15 D_s$$

(where T is elapsed time in weeks

D_p is the exponent of the peak titer in the expression
(25×2^{D_p})

D_s is similarly the exponent of the surveillance titer)

Coefficient of multiple determination 0.19
Coefficient of multiple regression 0.44
Degrees of freedom 235, $p < 0.0005$

Figure 5 illustrates the regression as decay lines for given peak titers. The standard error of the estimate is 0.33. The estimate of persistence of antibody at any given titer is shown by the horizontal portion of the decay lines.

For antibody to Gilliam the calculated regression is very similar:

$$\text{Log}_{10} T = 1.44 + 0.04 D_p - 0.13 D_s$$

Coefficient of multiple determination 0.38
Coefficient of multiple regression 0.62
Degrees of freedom, = 235, $p < 0.0005$

The mean peak log titers in relation to data points were 4.35 for the antibody to the polyvalent antigen and 4.27 for Gilliam due to uncontrolled weighting.

Discussion: The regression equations enable reversion rates of antibody to be calculated for given periods of time and from these a variety of estimates can be made. The calculations were performed using the regression of antibody to polyvalent antigen despite the better level of determination of the Gilliam regression, as we have unpublished evidence that over 90% of human infections in this area involve Karp/TA763/TA716 antigens while Gilliam is rare; antibody response to known antigens is non-specific and the mean peak-titer of the antibody to polyvalent antigen by regression data points is in much closer agreement with the actual mean peak titer than is the case for antibody to Gilliam antigen.

Table 1 shows the calculation of annual reversion using a calculated distribution of peak titer and Table 2 shows similarly the annual reversion for the observed distribution of peak titer. Correction to two significant figures gives an estimate of 0.61 by both methods.

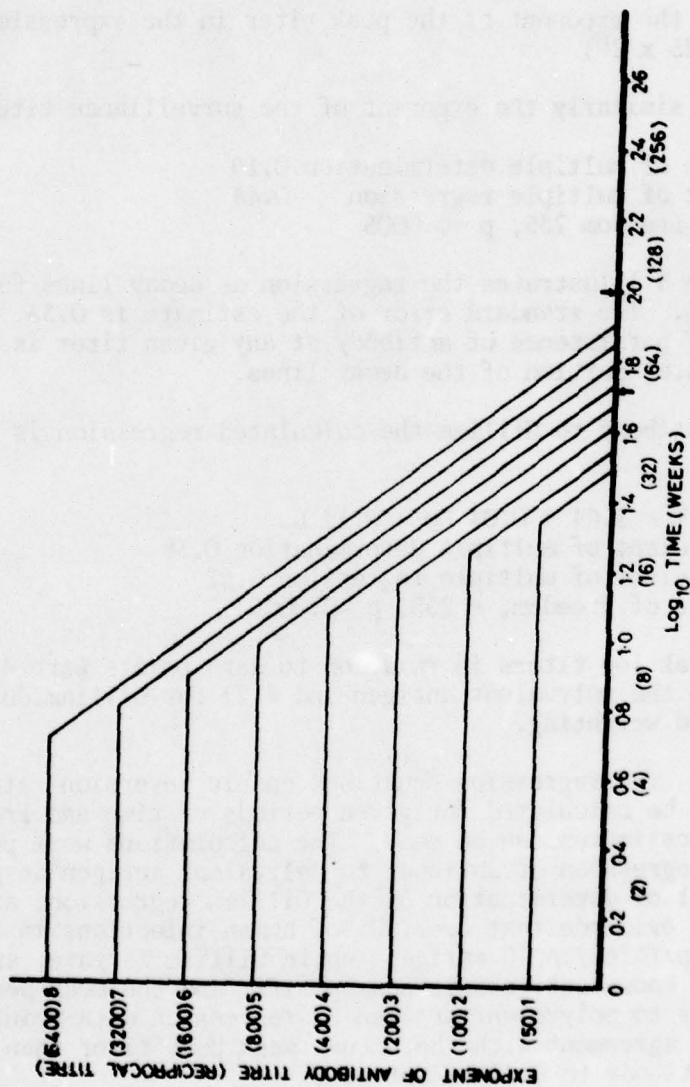


Figure 5. Persistence and decay of antibody to Karp/716/763 strains of *R. tsutsugamushi* as indicated by the calculated regression
 $\text{Log}_{10} \text{ Time} = 1.43 + 0.06 \text{ Dp} - 0.15 \text{ Ds}$.

Table 1. Annual reversion rate using calculated distribution of peak titer.

Peak Titer (reciprocal)	50	100	200	400	800	1600	3200	6400	Total
Proportion at peak	.020	.072	0.182	.326	.205	.139	.046	.010	1.00
Proportion reverting annually	.76	.73	.70	.63	.56	.49	.42	.35	NA
Total proportion reverting	.015	.053	.127	.205	.115	.068	.019	.003	.605

Table 2. Annual reversion rate using observed distribution of peak titer.

Peak titer (reciprocal)	50	100	200	400	800	1600	3200	6400	Total
Proportion at peak	.01	.05	.26	.28	.21	.04	.04	.02	1.00
Proportion reverting annually	.76	.73	.70	.63	.56	.49	.42	.35	NA
Total proportion reverting	.008	.037	.182	.176	.118	.064	.017	.007	.609

Table 3 shows the estimated proportion of a population initially showing antibody titer $\geq 1:50$ remaining positive at the elapsed times indicated. It is thus possible to separate the background contribution of residual antibody from the total prevalence in the course of longitudinal studies.

Attack rates can be estimated from point prevalence studies, if reversion rate is known, by the method of Muench (35) where, if

y = proportion positive
a = annual attack rate
b = annual reversion rate
t = time of exposure in years

$$\text{they } y = \frac{a}{a + b} (1 - e^{-t(a + b)})$$

(y approaches $\frac{a}{a + b}$ if b and/or t are large)

Using the prevalence rates found by Brown, Robinson and Huxsoll (4) the following results are obtained:

Pos Iskandar: Attack rate 0.55 p.a.

Expected infection for 516 man-months observation: 23.8
Definite infection: 20 (and an additional 18 classed as presumptive of which only 2 were probable)

Definite and probable infections: 22

Bukit Lanjan: Attack rate 0.70 p.a.

Expected infection for 216 man-months observation: 12.5
Definite infection: 7 (and an additional 4 classed as presumptive)

Definite and possible infection: 11

Using the data of Olson and Bourgeois (36) for Taiwanese military personnel on the Pescadores and, as exposure was limited to five months, the full Muench equation:

Attack rate = 0.14 p.a.
Expected infections = 25.6
Observed infections = 23

Thus in circumstances of presumed "steady state" between infection and reversion rates and in circumstances of limited exposure, the reversion rate calculated from our estimate of antibody longevity is accurately predictive of attack rates.

Table 3. Proportion of population with persisting antibody titer $\geq 1:50$.

Elapsed Time (months)	0	6	12	18	24	30	36	40	48	60
P	1.00	.63	.39	.25	.15	.10	.06	.05	.02	.01

Robinson, Gan and Donaldson (44) in part using the earlier work on CF antibodies by Shishido (48) suggested antibody persistence of 10-20 years in contrast to our finding of total reversion in 40 months (95% confidence level) and made a tentative estimate of a 0.03 annual attack rate in the rural Malaysian population on that basis and using a point prevalence of 0.35. Our findings suggest that the attack rate required to sustain such a prevalence is 0.32 p.a. and that for their group with the highest point prevalence (62% of Kelantan rubber tappers) an attack rate of 0.98 p.a. is indicated.

The population from which our sample was drawn is one subjected to a very high risk of infection (5) - it would be reasonable to describe the area as holoendemic for scrub typhus. The population is, therefore, subject to repeated infections. Additionally, the high incidence of other fevers (46) greatly increased the chances of non-specific anamnestic rise in pre-existing antibody to R. tsutsugamushi. As no attempt was made to select out data points that might have been affected by such factors - unless the primary criteria of scrub typhus appeared - the estimate of antibody longevity is probably too long and the variance somewhat increased.

Despite these possible confounding factors it is clear that human antibody to R. tsutsugamushi as measured by the indirect fluorescent antibody test is extremely short-lived. The regression equation derived from our data fits closely the observations made by other workers. It is thus also clear that the incidence of scrub typhus in rural Malaysia is much greater than was heretofore thought to be the case: indeed among agriculture sector workers (24) alone there could well be in excess of 500,000 cases of infection p.a. and nearly 200,000 cases of scrub typhus p.a. in contrast to the reported average 55 p.a. for the period 1967-1974 (5). Using conservative assumptions on length of illness, proportion actually affected by illness and number hospitalized, direct costs of this disease in agricultural sector workers will exceed M\$15 million annually. If other exposed workers and families were to be included in the estimate and indirect costs added, the bill could well exceed 3% of rural development expenditure.

SINGLE DOSE DOXYCYCLINE THERAPY FOR SCRUB TYPHUS

During the past year analysis of the data was completed, and the study has been reported (6).

The proposal to study hospitalized patients treated with single dose doxycycline and discharged early was not done since the study would have resulted in inefficient use of unit resources.

The local impact of the successful comparison of a single 200 mg oral dose of doxycycline with the conventional 7 day course of tetracycline for the treatment of scrub typhus, has lead to a more routine use of doxycycline by clinicians at our study sites. There is a tendency, which is not wholly consistent, for the clinician in charge to administer the drug earlier in the admission than therapy was given heretofore.

As one of the unresolved problems of single dose doxycycline therapy is the incidence of relapse with its administration early in the course of the disease we will analyse data on PUO trial patients (subsequently confirmed as suffering from scrub typhus) who received doxycycline.

Data collection began at the start of calendar 1978, and sites now include the rural health center at Jengka and the District Hospitals at Mentakab and Kuala Pilah. Data on all cases is not yet fully accessible (see ADP report), but from all sources there are about 60-80 patients who had scrub typhus and received early doxycycline. No relapses have been observed, but if the true relapse rate were as low as 1%, some 300 patients would need observation for a zero relapse count to achieve the 95% confidence level.

Observation continues and with indications that there is an increasing rate of early doxycycline usage directed by the clinicians in charge.

A STUDY OF THE CLINICAL, PATHOLOGICAL, EPIDEMIOLOGICAL AND THERAPEUTIC CHARACTERISTICS OF SCRUB TYPHUS AMONG FEBRILE PATIENTS AT AN ISOLATED RURAL HEALTH CENTER IN MALAYSIA

The purpose of this study is to establish the current picture of febrile illnesses - with particular emphasis on scrub typhus - among a susceptible population in areas of high risk and at an early stage of the disease. Studies among febrile patients presenting at rural hospitals and other studies at a FELDA settlement have shown FELDA oil palm settlers to be susceptible and at risk. However, the pattern of disease among hospital patients is likely to be very different from that at the level of the rural health center, and it is known that the average length of fever at the time of admission to hospital is 7-8 days. Also at the earlier studies at Bukit Mendi the alternative sources of health care delivery, other than the rural health center, were numerous and readily available, and it is probable that the limited population there is already beyond the susceptible period and (due to ecological changes) is not exposed to the same high level of risk. An area was sought, therefore, that overcame these problems and was

identified as the FELDA complex known as the Jengka Triangle in Western Pahang.

In addition to the advantages from the viewpoint of human studies, the complex (which consists of some 23 schemes of nearly 4000 acres each) offers a unique opportunity to study relevant ecology at all stages of development from primary jungle to established plantations and relate this to actual and concurrent human infection. There are sufficient schemes at an early enough stage of development for the complex to be the site for future trials of prophylactic agents, at which time the information gathered in this survey will be invaluable as a basis for prediction of expected infection/disease rates.

In essence the method of study used in the hospital based 'PUO study' is translocated to the rural health center in the middle of the complex, but certain modifications have been necessary to accommodate the local and logistic problems inherent due to the relative inaccessibility of the chosen site.

Study Coordination: This study is part of an interlocking series of studies being conducted in parallel. It is emphasized that this project is the main source of isolate material and sera. Most importantly it is supplying human origin specimens from the same areas where distribution of R. tsutsugamushi in vectors and reservoir hosts is being studied along with factors influencing transmission of the organism. It is the study by which much of the data obtained through various projects are integrated and together with the longitudinal study in the same area will enable interpretation of the mass of generated information in respect of human disease and infection.

Current Status: The study has been underway for some 17 months and despite the frequent re-assignment of the clinician in charge (the fourth incumbent is now in office), a high proportion of fever cases are referred through the unit laboratory. Complete analysis of the data is not yet possible as ADP soft-ware is incomplete, but there have been well over 400 PUO cases investigated and at least 103 isolates of R. tsutsugamushi obtained since the inception of the study (an additional 33 isolates were obtained from Jengka patients at Mentakab Hospital) and a considerable number of isolation results remain to be concluded.

Surprisingly, the pattern of diagnoses remains much the same as at district hospital level, except that (among adults) scrub typhus accounts for 60-80% of fevers. This finding is further confirmation of the validity of the estimations made as a result of the antibody longevity study. It is apparent also that indices of scrub typhus occurrence in the various schemes probably vary in accord with the postulates formed from the Bukit Mendi study.

The study continues in its present form except that routine isolation of R. tsutsugamushi is no longer carried out.

HOSPITAL BASED PUO STUDY

This study continues to be executed at the District Hospital Mentakab, Pahang. Additional information is obtained from samples forwarded by other hospitals (v.i.):

1. Mentakab: The format of the study remains unchanged. Patients have been admitted to the study at an average rate of approximately 40 monthly. A detailed breakdown of diagnostic categories is not currently possible due to the transfer to ADP format of recording data before the completion of ADP system. It is, however, clear that approximately 20% of cases are due to scrub typhus, some 10%+ due to leptospirosis and over 5% due to typhoid. By use of special logistic arrangements, patients admitted to the trial are currently the main source of specimens for CMI and monocyte culture studies.

2. Other Hospitals: The only hospitals contributing significant numbers of sera remain Seremban and Kuala Pilah, both in Negri Sembilan. In Seremban, the study is bedevilled by uncontrolled selection, personnel changes and lack of standardized information. The picture is further complicated by the hospital being a General Hospital and thus a point of secondary referral. Nevertheless the processing of samples from these two centers remains a useful monitor on the distribution of causal diagnoses of PUO cases within the general geographic area of the origin of patients in field studies. The initial efforts to standardize the acquisition and recording of data have been very successful. In Kuala Pilah the standard is identical to that at Mentakab, while at Seremban the data are standardized but acquisition is less detailed.

The re-assignment of the Consultant Physician at Mentakab to Teluk Anson in Perak State has resulted in a request by the district hospital at Teluk Anson to be involved in the PUO study. It is felt that this involvement would be contributory to the research effort and result in only a minimal increase in work. It is hoped that the arrangement will be formalized shortly.

A LONGITUDINAL STUDY OF THE CHARACTERISTICS OF R. TSUTSUGAMUSHI
INFECTION AMONG FELDA OIL PALM SETTLERS IN WEST PAHANG

This study was initiated in April 1977 using a carefully planned and detailed schedule.

There are two areas where information is required: firstly the comparison of disease, infection and recrudescence (or re-infection) rates to produce a realistic assessment of risk and secondly to relate infection and disease rates to influencing factors (e.g. ecological, meteorological, chigger species, chigger infectivity, organism strains etc.) to give some "index of risk". There is also the necessity to firmly identify not only a generic "high risk population" but also the prediction of the infection/disease pattern within that population when the time comes for human trials of prophylactic agents of any nature.

The studies at Bukit Mendi together with the limited extrapolation that can be made from some of the hospital-based PUO study material, would seem to indicate that the combination of a susceptible population and its location in a high risk area only prevails for a limited time (approx. 1-2 years) in the first 3 years of a FELDA oil palm settler's life. A new scheme was identified in the Jengka triangle suitable for longitudinal study.

The servicing of the study is amalgamated with the rural health center PUO study at Jengka. The study itself is in three phases.

1. A complete cross-sectional survey of settlers on two schemes within Jengka. One (Jengka 15) is an oil palm settlement of some 340 families (total population with dependents approx. 2000) on which settlement began in October 1976 and was complete by March 1977. It is expected that this population is susceptible and that the vector chiggers have not yet become fully established within the scheme. The initial serological, isolation, hematological and social data provide a base line against which to judge events and changes. The other (Jengka 11) is an oil palm settlement of some 450 families (total population over 3000) which is about 15 months older than Jengka 15. Approximately 350 settlers are 'original' and it is known that clinical cases of scrub typhus have occurred among them in recent months, and the remainder settled during the same period as the Jengka 15 population. In this settlement, therefore, there is a risk and part of the population is (probably) highly susceptible and part less so.

2. The Jengka 15 population has monthly sample surveys of approximately 15% of the population; selection is by random number (using the settler identification number as the index). Selection is exclusive. Information collected at each sample survey includes serological, hematological, isolation, and social/work data.

3. The Jengka 15 settlers are monitored at the daily roll call for sickness absence in an effort to detect all febrile illnesses and obtain a causal diagnosis. By this means and the random surveys the pattern of infection and disease with respect to scrub typhus will emerge.

Contemporaneously with these exercises, information concerning influencing factors is being gathered in other studies thus enabling the production of the risk index. Additionally, this is the first attempt to study and distinguish between infection and disease incidence in a population that eliminates (as far as is ever possible) uncontrolled effects of selection and competing health care delivery systems.

Current Status: Data collection has proceeded along the planned paths with subject compliance showing a slow decline from virtually 100% to the range 60-75% at the present. The survey is currently in the third six month cycle of sampling at Jengka 15.

Analysis of the data is not yet possible as the ADP soft-ware will not be fully operational until November 1978. It is clear, however, that the population of Jengka 15 showed evidence of only a low attack rate prior to the initial cross-sectional survey in April/May 1977 (prevalence of antibody by IFAT 9.4%, indicated attack rate 5.3% p.a.). No clinical cases occurred on Jengka 15 until October 1977 and since then there have been approximately 40 cases of fever attributed to scrub typhus. Human disease occurrence was compatible with the results of measurement of ecological factors.

On Jengka 11 prevalence of antibodies has indicated a far higher attack rate through the time of the field studies. This is reflected in the numbers of clinical cases (more than double those from Jengka 15) and correlates with the ecological findings.

The study continues on Jengka 15.

EPIDEMIOLOGY OF SCRUB TYPHUS ON A MALAYSIAN OIL PALM DEVELOPMENT

Although the first outbreak of scrub typhus among oil palm workers in Malaysia was described 50 years ago (16), it was only recently that the disease was identified as a leading cause of illness in rural Malaysia, particularly in oil palm workers (5). The infection is known to be distributed patchily in oil palm, dependent on localized ecological conditions (16). Therefore, it is essential to identify the conditions responsible for the high prevalence of infection. In addition, strain characterization of the isolates recovered from humans, rodents and mites is necessary

to determine the distribution of different antigenic types that exist in an endemic area.

This epidemiological study was undertaken on a scheme in the Federal Land Development Authority (FELDA) oil palm complex at Bukit Mendi. A detailed description of this study was included in the previous report (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977); therefore, only a brief description will be presented here. The scheme consisted of 3 phases, and at the beginning of the study, trees of Phases I and III were 7 and 5 years old respectively. On this scheme, as in all oil palm development schemes, settlers were not brought into the scheme until the trees approached bearing age, which was approximately 3 years after planting. They were provided with a house and approximately 10 acres of trees. Each family was responsible for the complete maintenance of his acreage, including weeding, fertilizing, pruning, and harvesting of fruit. Division of labor, as is often seen in private estates, was not established. All workers lived in Bukit Mendi village, with a total population of approximately 2500 during the time of study. The village contained a processing factory, offices, shop-houses, and schools, so all residents were not equally exposed to infection in the plantation.

1. Human Infections

A previous report illustrated the incidences of scrub typhus in relation to the site of exposure and by age groups and sex (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977). The largest incidence was seen among the workers in Phase III with a much reduced number in Phases I and II as well as in the village. Majority of cases were in people between the ages of 15 and 44, but the overall distribution of the disease was equal between sexes.

The strain characterization of the human isolates is shown in Table 4. No isolates contained the Gilliam antigen.

Final serum samples were obtained from the available settlers of the scheme. These represented specimens taken 1-3 years after the onset of the study. The results showed that 37% (40/108) and 13.6% (26/191) of the sera from Phases I and III respectively possessed scrub typhus antibodies.

2. Rodent and Chigger Infections

Last year, it was noted that the most common animal trapped in the study area was Rattus tiomanicus, comprising 75% (234/312) of the total animals (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977). Rickettsia tsutsugamushi organisms were isolated from 5 different animals:

Table 4. Strain characterization of human isolates from Bukit Mendi Scheme

Strain(s)	PHASES			
	I	II	III	Village
Karp				1
TA763	1			
Karp, TA716			1	
Karp, TA763				1
TA716, TA763		1	1	
Karp, TA686, TA716			1	
Karp, TA716, TA763				1
Karp, TA763, TH1817			1	
Karp, TA686, TA716, TA763			4	
Karp, TA716, TA763, TH1817		1	1	
Karp, Kato, TA763, TH1817			1	
Karp, TA686, TA716, TA763, TH1817			1	
Total	1	2	11	3

R. tiomanicus - 20.9% (49/234); R. argentiventer - 26.1% (6/23); R. exulans - 25.0% (1/4); Callosciurus notatus - 14.3% (2/14); and Tupaia glis - 11.1% (1/9) (Table 5). The FF grid was established on the forest fringe, so that equal number of traps were in the forest and the oil palm habitat. The YY grid was exclusively in the oil palm area. A greater number of different species of animals were collected in the FF grid, because of the diverse habitats existing within the area.

Results of serological characterization of the 59 isolates are shown in Table 6. Eight of the isolates were not recovered during reisolation attempts for strain characterization. Gilliam antigens were carried in 23.5% (12/51) of the isolates, while the Karp antigen and those related to the Karp antigen were found in 90.2% (46/51) of the isolates. The different antigenic strains appear to exist rather uniformly throughout the 2 grids of Phase III (Table 7). The breakdown of the strains among the different groups of animals from which the isolates were made showed no specific pattern (Table 8).

During the study, only 2 species of mites, Leptotrombidium (L.) deliense and L. (L.) vivericola, were collected by the black plate method. All but 2 L. (L.) vivericola chiggers were collected from Phase I, whereas 98.5% (1798/1825) L. (L.) deliense chiggers were from Phase III. Only one of 91 positive L. (L.) deliense chiggers was found in Phase I (Table 9). Of the remaining 90 positive chiggers found in Phase III, 18.9% (17/90) were collected in YY grid and 81.1% (73/90) in FF grid. Fifteen of 359 (4.2%) L. (L.) vivericola were infected with scrub typhus organisms.

Characterization of these organisms demonstrated that all reacted antigenically to the Karp and Karp-related strains, either singly or in combination with each other (Table 10).

SERO-EPIDEMIOLOGICAL SURVEY OF NEW ZEALAND FORCES SERVING IN SINGAPORE AND TRAINING IN MALAYSIA

The New Zealand forces stationed in Singapore consist largely of combat troops who remain for approximately 1 year and train intermittently in Johore State, Malaysia. The survey of these troops had two objectives - firstly to quantify the problem of scrub typhus and secondly to assess the populations for possible participation in field trials of prophylactic agents. The study takes the form of two cross-sectional sero-epidemiological surveys separated by about 6 months, during which time they train in Johore on several occasions for periods ranging from 3 days to 3 weeks.

The first paired surveys are complete. The results showed a conversion rate from <1:200 titer of Proteus OXK agglutination to

Table 5. Isolation of scrub typhus organisms from different animals trapped at Bukit Mendi oil palm scheme.

Animals	PHASE I (7)		PHASE III (FF)		PHASE III (YY)	
	Pos/Total*	% Pos	Pos/Total	% Pos	Pos/Total	% Pos
<u>Rattus tiomanicus</u>	2/41	4.9	32/118	27.1	15/75	20.0
<u>Rattus argentiventer</u>	-	-	1/9	11.1	5/14	35.7
<u>Rattus whiteheadi</u>	-	-	0/5	0	-	-
<u>Rattus muelleri</u>	-	-	0/4	0	-	-
<u>Rattus exulans</u>	-	-	1/1	100.0	0/3	0
<u>Rattus sabanus</u>	-	-	0/1	0	-	-
<u>Echinosorex gymmurus</u>	-	-	0/4	0	-	-
<u>Callosciurus notatus</u>	-	-	2/14	14.3	-	-
<u>Sundasciurus lowii</u>	-	-	0/1	0	-	-
<u>Chiropodomys gliroides</u>	-	-	0/1	0	-	-
<u>Sundasciurus tenuis</u>	-	-	0/12	0	-	-
<u>Tupaia glis</u>	0/2	0	0/6	0	1/1	100.0
Total	2/43	4.7	36/176	20.5	21/93	22.6

* Pos/Total = number of animals from which scrub typhus organisms were isolated/total number of animals.

Table 6. Antigenic strains of isolates from animals trapped at Bukit Mendi oil palm scheme.

Antigens	Animals	
	Number	%
Karp	10	19.6
Gilliam	5	9.8
Karp, Gilliam	2	3.9
Karp, TA686	4	7.9
Karp, TA763	12	23.5
Karp, TH1817	1	2.0
Gilliam, TA716	1	2.0
TA686, TA716	1	2.0
TA716, TA763	1	2.0
Karp, Gilliam, TH1817	1	2.0
Karp, TA686, TA716	1	2.0
Karp, TA686, TA763	4	7.9
Karp, TA716, TA763	1	2.0
Karp, TA763, TH1817	2	3.9
Gilliam, TA716, TA763	2	3.9
TA686, TA716, TA763	2	3.9
Gilliam, TA686, TA716, TA763	1	2.0
Total	51	100.3

8/59 isolates were not recovered during the attempt to reisolate the organisms for characterization.

Table 7. Antigenic strains of isolates from animals trapped in different grids at Bukit Mendi oil palm scheme.

Antigens	PHASE I (7)		PHASE III (FF)		PHASE III (YY)	
	Number	%	Number	%	Number	%
Karp	-	-	6	20.0	4	21.1
Gilliam	1	50	2	6.7	2	10.5
Karp, Gilliam	-	-	1	3.3	1	5.3
Karp, TA686	-	-	3	10.0	1	5.3
Karp, TA763	1	50	7	23.3	4	21.1
Karp, TH1817	-	-	-	-	1	5.3
Gilliam, TA716	-	-	1	3.3	-	-
TA686, TA716	-	-	1	3.3	-	-
TA716, TA763	-	-	1	3.3	-	-
Karp, Gilliam, TH1817	-	-	1	3.3	-	-
Karp, TA686, TA716	-	-	1	3.3	-	-
Karp, TA686, TA763	-	-	3	10.0	1	5.3
Karp, TA716, TA763	-	-	1	3.3	-	-
Karp, TA763, TH1817	-	-	1	3.3	1	5.3
Gilliam, TA716, TA763	-	-	-	-	2	10.5
TA686, TA716, TA763	-	-	1	3.3	1	5.3
Gilliam,TA686,TA716,TA763	-	-	-	-	1	5.3
Total	2	100	30	99.7	19	100.3

Table 8. Antigenic strains of isolates from different animals trapped at Bukit Mendi oil palm scheme.

Antigens	<u>R.tiomanicus</u>		<u>R.argentiventer</u>		<u>R.exulans</u>		<u>Tupaia glis</u>	
	Number	%	Number	%	Number	%	Number	%
Karp	8	17.8	1	25	-	-	1	100
Gilliam	5	11.1	-	-	-	-	-	-
Karp, Gilliam	1	2.2	1	25	-	-	-	-
Karp, TA686	4	8.9	-	-	-	-	-	-
Karp, TA763	10	22.2	2	50	-	-	-	-
Karp, TH1817	1	2.2	-	-	-	-	-	-
Gilliam, TA716	1	2.2	-	-	-	-	-	-
TA686, TA716	1	2.2	-	-	-	-	-	-
TA716, TA763	1	2.2	-	-	-	-	-	-
Karp, Gilliam, TH1817	1	2.2	-	-	-	-	-	-
Karp, TA686, TA716	1	2.2	-	-	-	-	-	-
Karp, TA686, TA763	3	6.7	-	-	1	100	-	-
Karp, TA716, TA763	1	2.2	-	-	-	-	-	-
Karp, TA763, TH1817	2	4.5	-	-	-	-	-	-
Gilliam, TA716, TA763	2	4.5	-	-	-	-	-	-
TA686, TA716, TA763	2	4.5	-	-	-	-	-	-
Gilliam, TA686, TA716, TA763	1	2.2	-	-	-	-	-	-
Total	45	100.0	4	100	1	100	1	100

Table 9. Identification of scrub typhus organisms in L. (L.) deliense and L. (L.) arenicola chiggers collected at Bukit Mendi oil palm scheme in 1976/1977.

Month	<u>L. (L.) deliense</u>		<u>L. (L.) vivericola</u>	
	Pos/Total*	% Pos	Pos/Total	% Pos
Mar 76	4/90	4.4	0/0	0
Apr	1/91	1.1	0/0	0
May	20/445	4.5	0/40	0
Jun	-**	-	-	-
Jul	-	-	-	-
Aug	1/58	1.7	0/0	0
Sep	0/0	0	0/0	0
Oct	10/223	4.5	0/95	0
Nov	3/113	2.7	2/70	2.9
Dec	10/134	7.5	4/32	12.5
Jan 77	7/131	5.3	7/105	6.7
Feb	4/67	6.0	0/0	0
Mar	20/351	5.7	2/16	12.5
Apr	2/20	10.0	0/1	0
May	0/11	0	0/0	0
Jun	7/31	22.6	0/0	0
Jul	2/60	3.3	0/0	0
Total	91/1825	5.0	15/359	4.2

* Pos/Total = Number of chiggers in which scrub typhus organisms were identified/total number of chiggers.

**No samples were collected for identification.

Table 10. Antigenic strains of *R. tsutsugamushi* organisms identified in chiggers collected at Bukit Mendi oil palm schemes.

Antigens	<u>L. (L.) deliense</u>		<u>L. (L.) vivericola</u>	
	Number	%	Number	%
Karp	62	68.1	13	86.7
TA763	6	6.6	-	-
Karp, Kato	2	2.2	-	-
Karp, TA686	2	2.2	-	-
Karp, TA716	1	1.1	-	-
TA716, TA763	1	1.1	-	-
Karp, TA686, TA716	1	1.1	-	-
Karp, TA716, TA763	1	1.1	-	-
Karp, TA686, TA716, TA763	15	16.5	2	13.3
Total	91	100.0	15	100.0

>1:200 titer of nearly 15%. There was, however, a zero conversion rate for R. tsutsugamushi antibody as measured by IFAT. Conversion rates in these two tests in earlier studies of expatriate troops are being examined to enable interpretation of this result.

The second paired surveys are currently incomplete.

CHARACTERIZATION OF "UNTYPED" STRAINS OF RICKETTSIA TSUTSUGAMUSHI FROM HUMAN PATIENTS

The 3 strains were placed in the "untyped" category, when initial attempts to characterize the strains by direct fluorescent antibody test demonstrated the lack of reaction between the isolate organisms and the specific conjugates prepared against the 8 prototype strains. Subsequent study involving the use of specific immune sera of these isolates in the indirect fluorescent antibody test against the prototype and their own antigens resulted in incomplete crossing, making the analysis difficult. Therefore, the efforts to study the antigenic make-up of these isolates will be curtailed until another test procedure is found to adequately analyze the existing strains.

STUDIES OF CELL MEDIATED IMMUNITY IN RICKETTSIA TSUTSUGAMUSHI INFECTION

During the past year, 258 specimens from PUO patients were collected for cell mediated immunity (CMI) studies. Blast transformation studies of lymphocytes from 50.4% (130/258) human samples could not be performed due to 3 main factors: defective equipment, contamination, and insufficient cell density.

Lymphocytes from 49.6% (128/258) patients were examined for stimulation by phytohemagglutinin (PHA) and specific antigens. Despite the use of varied preparations of specific scrub typhus antigens, including either-extracted, sonicated, homogenized, heat-inactivated, formalin-treated, KCI-extracted, and different sucrose density gradient fractions, specific stimulations of the cells were not observed. Samples from the specimens used to culture the lymphocytes were also examined concurrently for the presence of scrub typhus organisms and antibodies. Eighteen of 128 (14.1%) specimens had both organisms and antibodies. Scrub typhus rickettsiae were isolated from 5 (3.9%) of the samples which did not show any antibodies, while 28 (21.9%) specimens contained demonstrable antibodies but no rickettsiae.

Although a specific CMI response was not demonstrated with the R. tsutsugamushi antigens tested, the success of culturing lymphocytes despite the existing logistic problems cannot be overlooked. All specimens were obtained from a district hospital located 80 miles from Kuala Lumpur. They were placed in a container filled with ice, and transported to the laboratory in Kuala Lumpur by a taxi. The trip took a minimum of 3 hours and sometimes 6 hours, depending on the traffic.

Upon receipt of the samples, the lymphocytes were separated in lymphocyte separation medium and by series of low speed centrifugations. The lymphocyte cultures were incubated with PHA for 3 days, after which they were pulsed for 16 hours. The cells were harvested with a multiple cell harvester, and the isotope incorporation was assayed by liquid scintillation counting. It was shown that most cells responded to the non-specific stimulant, despite the long time interval between the collection and the processing of the specimens.

Concerted efforts will be directed towards the development of an adequate scrub typhus antigen to measure the specific responses of the sensitized lymphocytes. Since the response may be dependent upon the amount of antigen present, an attempt to prepare antigens with higher infectivity titers will be made. If this is achieved, antigens, prepared by methods mentioned above as well as other methods, will be tested. This work will be performed utilizing the peripheral cells from experimentally infected mice and monkeys, and should the results be successful, the assay will be applied to lymphocytes from human patients.

INVESTIGATION OF NATURALLY OCCURRING STRAINS OF R. TSUTSUGAMUSHI FOR POTENTIAL USE AS A VACCINE

In vaccine development, consideration must be given to isolation and identification of attenuated strains with antigenic characteristics similar to those of the predominant strain existing in a particular geographic area. Studies in Thailand, West Pakistan, and Malaysia have demonstrated the predominance of the Karp and Karp-related strains (15; 47; USAMRU, unpublished data).

The intraperitoneal inoculation of the Karp strain usually results in death of susceptible outbred mice. However, isolates that resemble Karp serologically have been found in Malaysia that cause no deaths in laboratory mice. All infected mice survived a lethal challenge of the Karp strain. Seed suspensions for these isolates were prepared from infected mouse tissues. All produced very low infectivity titers of approximately $10^{-3.5}$ when titrated in normal laboratory mice. Because of this, further tests in mice and monkeys were curtailed.

THE IMPACT OF ECOLOGICAL CHANGES ON THE POPULATION OF SCRUB TYPHUS
VECTORS IN AN AREA CLEARED OF FOREST AND REPLANTED IN A TYPE OF
VEGETATION THAT NATURALLY SUPPORTS A CHIGGER POPULATION

Scrub typhus has long been associated with specific types of habitat. The name scrub typhus was coined to associate the disease with the scrub terrain where the disease was often contracted. Within Peninsular Malaysia, the vector mites which transmit the disease can be found in lalang fields, along sandy beaches, in different forest types and within major agricultural crops.

Since the early 1960's hundreds of thousands of acres of land in Malaysia have been put into agricultural production, primarily through large government-financed programs. The Federal Land Development Authority (FELDA), established in 1956, is now one of the largest land development agencies of the Malaysian Government. Based on a fully integrated approach, FELDA's operations include land clearing; planting of main crops (primarily oil palm and rubber); development of villages; selection and emplacement of settlers; management of projects; provision of credit; marketing services; and facilitating social and community development.

During the development stage FELDA contractors carry out jungle clearing, plant the main crop, and maintain the crop until settlers enter the project. Settler houses are also built by FELDA contractors. Roads, water supplies, schools and clinics are constructed by the Public Works Department. Settlers enter the project 2½ - 3 years after the start of land clearing and are immediately responsible for weeding, fertilizing, pest and disease control. Prior to the time that the settler receives income from his land, FELDA provides subsistence credits to the settler. Approximately 5 years after planting of oil palm (later in the case of rubber) the settler commences repaying the loan which covers the cost of land development, fertilizers, subsistence credits and a house. The settler acquires title to the land (or a share in a cooperative) after about 15 years of loan payment.

FELDA oil palm development schemes each consist of approximately 4000 acres. Approximately 400 settler families enter each scheme with 10 acres of oil palm allotted to each settler family.

The oil palm trees are planted in rows approximately 6 meters apart. Before the trees begin to bear fruit, grasses are allowed to grow around the trees. In order to harvest the fruit, a frond below the fruit must be cut. These fronds are stacked between every other row of trees (Figure 6). Thus, rows are separated alternately by grass strips and piles of fronds. As the settlers begin working their plot, some of the grasses are eliminated due to cultivation practices. Also, as the trees grow a canopy forms reducing the sunlight which reaches the grass around the trees. By this means many of the grasses are eliminated.

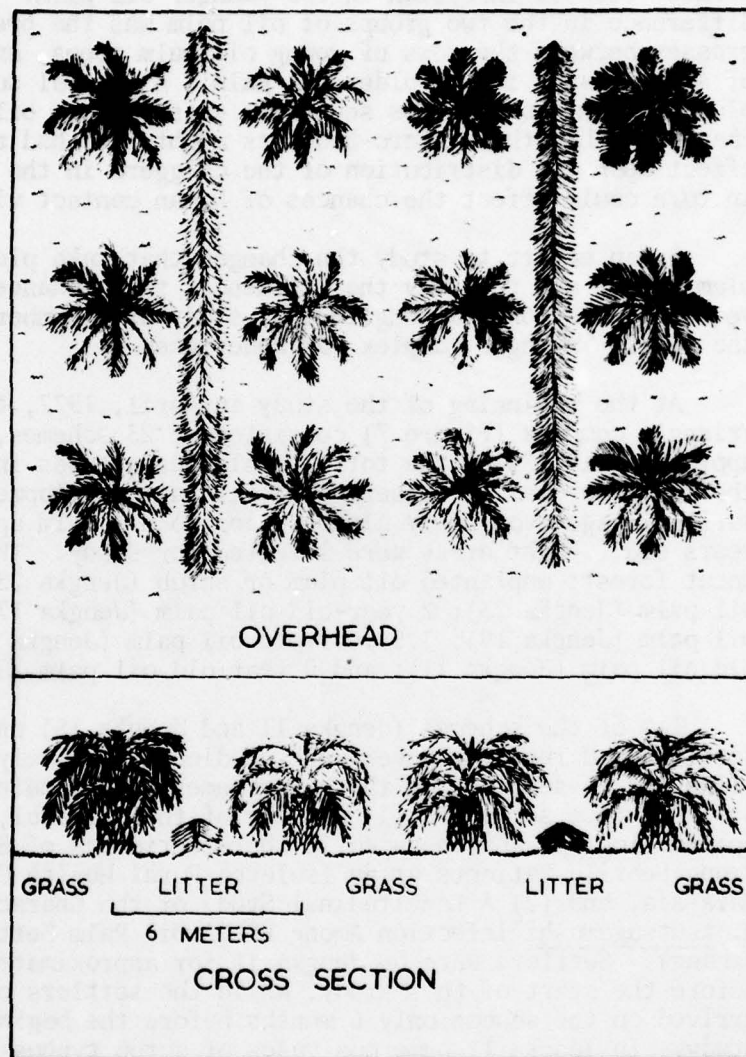


Figure 6. Schematic diagram showing the system of maintaining trees and litter piles within a Federal Land Development Authority oil palm scheme.

Previous studies at a district hospital and a rural health center provided data which showed that scrub typhus was a common cause of fever among oil palm workers (5). Studies at the Bukit Mendi FELDA oil palm development scheme, which contained plantings of 5 and 7 years of age, provided data which served to suggest a greater risk of infection in the younger oil palm. The major difference in the two groups of oil palm was the presence of dense grasses between the rows of young oil palm trees, and the absence of such grasses in the older oil palm. A natural succession of plants and micro-habitats seemed to exist in the oil palm. The changes within these micro-habitats might have had a definite effect upon the distribution of the chiggers in the areas, which, in turn could affect the chances of human contact with the vectors.

In an effort to study the changes that take place in an oil palm scheme and to study the influences these changes have upon vector populations, a longitudinal study of a number of schemes in the Jengka Triangle complex was undertaken.

At the beginning of the study in April, 1977, the Jengka Triangle complex (Figure 7) consisted of 23 schemes, of which approximately 63% of the total usable acreage was in oil palm and the remainder was in rubber. The oil palm development schemes varied in age from newly cleared land to oil palm approximately 10 years old. Eight areas were selected for study. These included: uncut forest; unplanted oil palm or scrub (Jengka 23); 1.5 year-old oil palm (Jengka 23); 2 year-old oil palm (Jengka 17); 2.5 year-old oil palm (Jengka 19); 3.5 year-old oil palm (Jengka 15); 5.5 year-old oil palm (Jengka 11); and 9 year-old oil palm (Jengka 2).

Two of the schemes (Jengka 11 and Jengka 15) on to which settlers had recently moved were studied intensively. Febrile illnesses in settlers on these 2 schemes were studied in other projects (see sections: (1) A Study of the Clinical, Pathological, Epidemiological and Therapeutic Characteristics of Scrub Typhus Among Febrile Patients at an Isolated Rural Health Center in Malaysia, and (2) A Longitudinal Study of the Characteristics of *R. tsutsugamushi* Infection Among FELDA Oil Palm Settlers in West Pahang). Settlers were on Jengka 11 for approximately 18 months before the start of this study, while the settlers on Jengka 15 had arrived on the scheme only 6 months before the beginning of the study. In Jengka 11 numerous cases of scrub typhus had been diagnosed, whereas in Jengka 15 cases had not occurred.

In an effort to correlate chigger populations with human and rodent infections, monthly collections of rodents and chiggers were made from Jengka 11 and Jengka 15. In each scheme two permanent trapping grids of 100 traps, set 6 meters apart, were established. Chiggers were collected from trapped rodents and by the black plate technique from within these grids. Additionally, to obtain samples from a larger area throughout these schemes, a "roving"

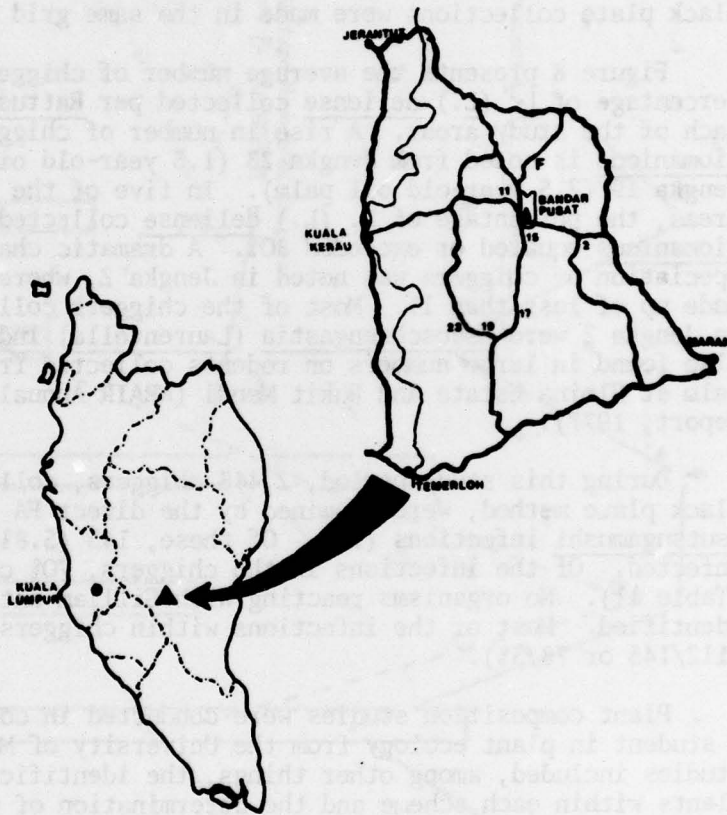


Figure 7. Jengka Triangle Complex, Pahang, Peninsular Malaysia, showing locations of the schemes under study.

Table 11. Characterization of Rickettsia tsutsugamushi strains from chiggers collected from Jengka Triangle study sites (March 1977 - March 1978).

Strains	Number	Percent
Karp	100	69.9
TA686	1	.7
TA716	4	2.8
TA763	7	4.9
Karp, TA686	6	4.2
Karp, TA716	8	5.6
Karp, TA763	8	5.6
TA686, TA763	1	.7
TA716, TA763	6	4.2
Karp, TA686, TA716	1	.7
Karp, TA716, TA763	1	.7
Total	143	100.0

CHARACTERIZATION OF RICKETTSIA TSUTSUGAMUSHI STRAINS
PRESENT IN INFECTED LABORATORY-REARED L. (L.) ARENICOLA
AND L. (L.) FLETCHERI CHIGGERS

In previous studies natural infections of silvered leaf monkeys by laboratory-reared L. (L.) arenicola and L. (L.) fletcheri chiggers, infected with R. tsutsugamushi, produced disappointing results as far as the development of any clinical signs (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977). In addition, the intradermal inoculation of monkeys with R. tsutsugamushi strains recovered from both species of chiggers resulted in poor clinical responses. The Arenicola strain was more virulent for mice, however, than the Fletcheri strain.

Recently, a fluorescent antibody (FA) technique was developed for identification of R. tsutsugamushi infections in chiggers (12). With this technique, which also can be used to type antigenically the infecting organisms, routine random examination of L. (L.) fletcheri colony chiggers showed differences in the antigenic make-up of the strains existing in different lines of chiggers from the same generation. To determine the true nature of the strains existing in both L. (L.) arenicola and L. (L.) fletcheri colonies, an extensive study was undertaken where chiggers from different lines from each colony were examined. From each line, 5 chiggers were harvested and examined by the FA technique. In addition, 5 chiggers were individually fed on mice, which were then observed for 28 days for morbidity and mortality. Another 5 chiggers were also fed individually on mice, but when these mice became ill, or on day 14 if not ill, they were killed and suspensions of livers and spleens were prepared and inoculated into 10 normal mice. These animals were observed for 28 days. All surviving mice were challenged with 10^3 MLD₅₀ of the virulent Karp strain. In addition, glycogen-treated mice (45) were inoculated with suspensions from the above mice, and peritoneal fluids were then examined for rickettsial organisms by direct immunofluorescence.

Examination of 23 different lines of L. (L.) arenicola chiggers by the FA technique demonstrated the following: 8 lines carried TA716 and TA763 antigens; 11 lines carried TA716, TA763 and TA686 antigens; and 4 lines carried TA716, TA763 and Karp antigens. Peritoneal exudates from mice on which the siblings of the above chiggers had fed are currently being examined. Although the results are incomplete, a greater expression of antigenic types has been noted when organisms in the infected L. (L.) arenicola chiggers were passed in mice.

In the case of L. (L.) fletcheri chiggers, those from 2 lines had TA716 and TA763 strains, while those from 3 lines carried only the Gilliam strain. The remaining chiggers demonstrated combinations of Gilliam with Karp-related (TA686, TA716, TA763, Karp, Kato) strains. The peritoneal fluids from mice on which L.

L. (L.) fletcheri chiggers had fed showed multiple antigenic strains, including both Gilliam and Karp-related strains.

Because the L. (L.) fletcheri chiggers contained organisms with 3 antigenic patterns (Gilliam; Karp-related; Gilliam and Karp-related), it was decided to examine the chiggers of the next generation from some of these same 22 lines and to determine if antigenic alterations occurred. Of 15 chiggers tested from Gilliam parents, 13 carried both Gilliam and Karp-related antigens; 1 demonstrated only TA716; and 1 showed Gilliam only. All chiggers tested from parents carrying only Karp-related strains demonstrated only Karp-related antigens. In the case of 10 chiggers tested from parents carrying both Gilliam and Karp-related strains, 7 showed both antigenic types; 2 carried Gilliam antigens only; and 1 showed only TA716 antigen.

The virulence of the chigger strains for mice was studied. It was noted that there were minimal illnesses and deaths in the mice that were fed upon by either species. Randomly-selected mice from these groups were also harvested 10-14 days after chigger induced infection. Tissue suspensions of livers and spleens were inoculated IP into normal mice. A high mortality (83%) and long duration of illness (10 days) were observed in those mice infected with tissue suspensions prepared from Arenicola-infected mice. Whereas sporadic deaths (22%) and shorter duration of illness (6 days) occurred in animals infected with suspensions from Fletcheri-infected mice.

LEPTOTROMBIDIUM (LEPTOTROMBIDIUM) VIVERICOLA, VERCAMMEN-GRANDJEAN AND LANGSTON, 1976, AS A POSSIBLE VECTOR OF SCRUB TYPHUS IN PENINSULAR MALAYSIA

Three species of Leptotrombidium are known to be vectors of scrub typhus in Peninsular Malaysia. These include: L. (L.) fletcheri (formerly akamushi in Malaysia, L. (L.) deliense and L. (L.) arenicola.

L. (L.) fletcheri is found in lalang fields (Imperata cylindrica) (22) throughout Peninsular Malaysia. The natural transmission of Rickettsia tsutsugamushi has been studied within an infected laboratory-reared colony of L. (L.) fletcheri (38; 40; 60). L. (L.) deliense is distributed throughout much of Southeast Asia and is the major vector in forest, scrub and oil palm habitats throughout Peninsular Malaysia (22; Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977). L. (L.) arenicola is found along the sandy beaches of Peninsular Malaysia (52; 56) and has been reported from beaches around Jakarta, Indonesia (19). Transovarial transmission has been reported from two separate laboratory-reared colonies of this species (37; 45).

As early as 1976, a species of chigger was collected that was taxonomically similar to L. (L.) arenicola (Walter Reed Army Institute of Research Annual Progress Report, 1 July 1975 - 30 June 1976). This species was collected from inland locations that were not associated with sandy beaches. Additional specimens of this species were collected from various study sites (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977). Recently, Vercammen-Grandjean and Langston (57) described a species, L. (L.) vivericola, which taxonomically conformed to this species.

A small number of L. (L.) vivericola from black plate collections at Elmina Estate and Bukit Mendi (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977) study sites were found to be infected. During more recent collections from Jengka, 12 of 885 (1.4%) L. (L.) vivericola chiggers were found to be infected. Two additional collections from Selangor (See Section: A Study of Rickettsia tsutsugamushi Infections in Chiggers Collected in Selangor, Peninsular Malaysia) containing a large number of this species did not have infected chiggers.

Of 4,876 chiggers collected by the black plate technique from the Jengka study sites and identified during the past year, 1,020 or 20.9% were L. (L.) vivericola. However, of the chiggers collected from mammals, less than one percent have been this species. The majority of the previous collections of L. (L.) vivericola from mammals have come from the tree shrew, Tupaia glis (Table 12). In comparison to previous collections, particularly Bukit Mendi, few T. glis have been collected from the Jengka study site. In an effort to determine the maintaining hosts for L. (L.) vivericola within Jengka, a single collection of birds was made. The majority of the birds netted were not ground-dwelling birds. However, of 20 chiggers collected from 3 chigger-infested birds, 18 (90%) were L. (L.) vivericola. Additional collections of ground-dwelling birds are planned to determine the extent of birds as hosts of this species.

During the past year, one collection was made from an area in Bukit Mendi that was presumed to have infected L. (L.) vivericola with the intention of establishing an infected colony. However, none of the chiggers examined proved to be infected. A more recent collection from presumably infected "mite islands" in Jengka is currently under study. Reared adults have been obtained and their offspring will be identified and tested for infectivity.

Table 12. Leptotrombidium (L.) vivericola collected from mammals from three study sites in Peninsular Malaysia.

Location and Mammals Species	Number L. (L.) <u>vivericola</u> Infested Mammals	Number Chiggers Identified	L. (L.) <u>vivericola</u> Number	
Taman Negara <u>Tupaia glis</u>	2	429	2	0.4
Elmina Estate <u>Tupaia glis</u>	3	134	3	2.2
<u>Rattus tiomanicus</u>	2	32	2	6.2
Bukit Mendi <u>Tupaia glis</u>	52	1300	251	19.3
<u>Rattus tiomanicus</u>	5	95	5	5.3
<u>Rattus argentiventer</u>	2	54	2	3.7

A STUDY OF RICKETTSIA TSUTSUGAMUSHI INFECTIONS IN CHIGGERS
COLLECTED IN SELANGOR, PENINSULAR MALAYSIA

Recently a technique for assaying *R. tsutsugamushi* infections in chiggers collected by the black plate method was described (12). Since that time most studies of *R. tsutsugamushi* infections in chiggers in Malaysia have been limited to chiggers collected from oil palm study sites in central Pahang. From these studies it can be generally concluded that the prevalence of infections in chiggers varies with collection sites; the strains in chiggers are similar to those recovered from rodents and patients in the same area; and while multiple antigens are detected in rodent and human isolates, the apparent infections in chiggers tend to be more monotypic with regard to antigenic strains.

Since conclusions drawn from examinations of chiggers collected from limited habitats in central Pahang cannot be applied, without considerable caution, to wider endemic areas, a study was recently undertaken to examine chiggers collected from other areas and habitats in Malaysia.

In the study reported here chiggers were collected during April and May 1978 by the black plate technique from 2 habitats, lalang and secondary forest, within a 30 mile radius of Kuala Lumpur (Figure 9). Three sites were in lalang fields (Rawang (1)), Kuala Selangor Road (2) and Jenderam (5)) and three sites were in secondary forests (Subang (3), Bangi (4) and Ulu Langat (6)).

Four species of chiggers belonging to the subgenera *Leptotrombidium* (*Leptotrombidium*) were collected during this study. These included: *L. (L.) fletcheri*; *L. (L.) deliense*; *L. (L.) vivericola*; and *L. (L.) bodense*. *L. (L.) fletcheri* and *L. (L.) deliense* are well known vectors of scrub typhus and *L. (L.) vivericola* is a recently described species that may be a possible vector of scrub typhus. (See Section: *L. (L.) vivericola* as a Possible Vector of Scrub Typhus). *L. (L.) bodense*, while never being incriminated as a vector, is taxonomically closely related to *L. (L.) fletcheri* and *L. (L.) deliense*.

A total of 998 unengorged chiggers were collected, Table 13. From the 3 lalang areas, the predominant species collected was *L. (L.) fletcheri*. Only a single specimen of *L. (L.) deliense* was collected.

Within the secondary forested areas, a wider variety of species was found. In the Subang study site predominant species were *L. (L.) deliense* (62.6%), and *L. (L.) vivericola* (30.8%). Also collected was a small number of *L. (L.) bodense* (6.6%). Surprisingly, the main species within the other two secondary sites, Bangi and Ulu Langat, was *L. (L.) vivericola*, with 98.6 and 99.5% respectively. Six (3.2%) *Babiania parvifera* were collected from Bangi and a single *L. (L.) deliense* was collected from Ulu Langat.

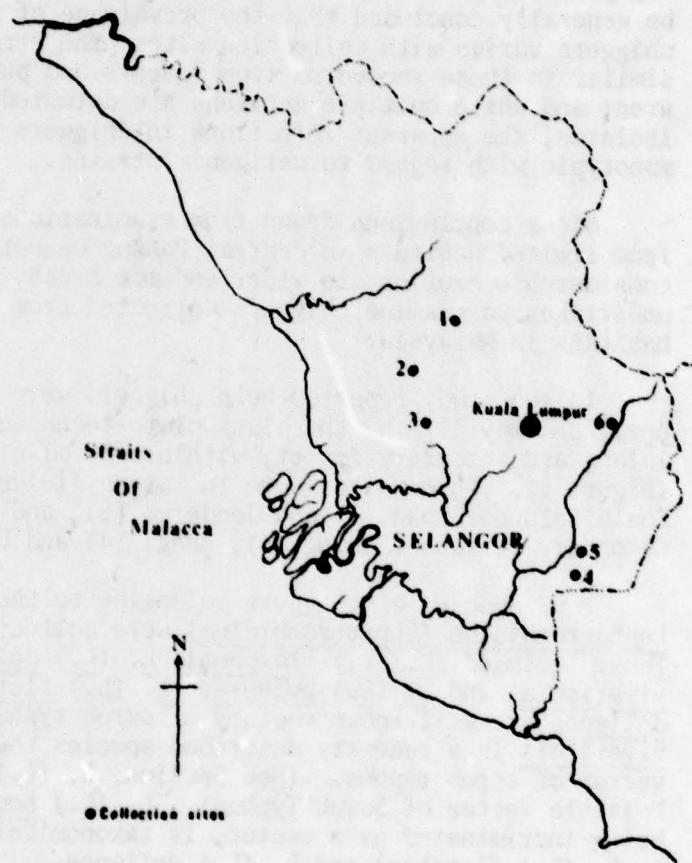


Figure 9. Locations of black-plated chigger collection sites in Selangor, Peninsular Malaysia
 (1) Rawang, (2) Kuala Selangor Road, (3) Subang,
 (4) Bangi, (5) Jenderam, (6) Ulu Langat.

Table 13. Trombiculid larvae collected from black plates from 6 localities within Selangor, Peninsular Malaysia.

Locality and Habitat	Total Number of Chiggers Collected	Chigger Species			Others
		<u>Leptotrombidium (Leptotrombidium) deliense</u>	<u>Leptotrombidium (Leptotrombidium) fletcheri</u>	<u>Leptotrombidium (Leptotrombidium) vivericola</u>	
<u>Lalang</u>	75	1	74	0	0
Rawang	141	0	141	0	0
K. Selangor Road	207	0	207	0	0
Jenderam					
<u>Secondary Forest</u>					
Subang	195	122	0	60	13*
Bangi	189	0	0	183	6 ⁺
Ulu Langat	191	1	0	190	0
Total	998	124	422	433	19

* Leptotrombidium (Leptotrombidium) bodense

+ Babangia parnifera

Out of the 998 chiggers collected, 850 (85.2%) were examined for R. tsutsugamushi by direct FA technique. The remaining 148 specimens were dead at the time of examination.

Within the 392 specimens examined from Ialang sites, 13 (3.3%) were infected: Rawang 3/71 (4.2%); Kuala Selangor Road 4/133 (3.0%) and Jenderam 6/188 (3.2%). Of the 13 infected chiggers, 12 reacted with the Karp strain. A single specimen from Jenderam reacted with the TA763 strain.

No organisms were observed in any of the chiggers collected from the secondary forest sites.

A STUDY OF RICKETTSIA TSUTSUGAMUSHI INFECTIONS IN CHIGGERS COLLECTED IN THAILAND

Antigenic heterogeneity of Rickettsia tsutsugamushi has been reported sufficiently for the trait to become widely accepted as characteristic of the organism. A study in Malaya showed the presence of the multiplicity of antigenic types in a single small area (28). The 8 strains isolated from mites and volunteers belonged to 4 sero-groups as measured by cross serum neutralization tests. Antigenic characterization of many isolates from Thailand, West Pakistan and Malaysia have demonstrated the predominance of Karp and Karp-related strains (15; 47; USAMRU, unpublished data). Knowledge of the prevalence and distribution of antigenic strains of R. tsutsugamushi in the endemic region is an obvious prerequisite to the selection of antigens in the development of immunoprophylactic agents.

Recently an immunofluorescence test was developed for the identification of R. tsutsugamushi infections in unengorged chiggers collected by the black plate technique (12). The test can also be used to antigenically characterize the infecting organism. This technique affords an efficient means of acquiring data on the prevalence and distribution of antigenic strains in selected habitats and geographic areas. Using this technique much data has been gathered from chiggers collected at study sites in central Pahang, Peninsular Malaysia, where Karp and Karp-related strains were found as the predominant antigens (Table 14). However, findings in central Malaysia may not reflect the prevalence and distribution of antigenic types in the entire endemic region; therefore, analysis of R. tsutsugamushi strains from wider geographic areas is essential. To this end, isolates from selected habitats throughout Peninsular Malaysia are being antigenically characterized, and arrangements have been made to acquire specimens for study from other areas in the Asiatic-Pacific region e.g. Thailand.

Table 14. Antigenic characteristics of Rickettsia tsutsugamushi organisms identified in chiggers from three different study sites in Peninsular Malaysia.

	Elmina Estate	Bukit Mendi	Jengka
Total specimens examined	664	2184	4418
Total positive specimens	11	106	179
Strains			
Karp	10	75	125
TA686	-	-	1
TA716	1	-	7
TA763	-	6	9
Karp, Kato	-	2	-
Karp, TA686	-	2	7
Karp, TA716	-	1	10
Karp, TA763	-	-	10
TA686, TA763	-	-	1
TA716, TA763	-	1	6
Karp, TA686, TA716	-	1	1
Karp, TA716, TA763	-	1	2
Karp, TA686, TA716, TA763	-	17	-

The first clinical case of scrub typhus in Thailand was described by Thaineua (51), and the first isolation of the rickettsial organism was from rodents (53). Subsequent investigations resulting in isolations of R. tsutsugamushi organisms from humans, rodents, and chiggers throughout the major physiogeographic areas have demonstrated the widespread occurrence of scrub typhus in Thailand (15; 54; 55; SEATO Medical Research Laboratory, Bangkok, Thailand, Annual Progress Reports, 1966, 1967, 1968, 1969).

Although 3 vector species of chiggers, Leptotrombidium (Leptotrombidium) deliense, L. (L.) scutellare and L. (L.) fletcheri are known to be present in Thailand, R. tsutsugamushi has been isolated only from L. (L.) deliense (SEATO Medical Research Laboratory, Bangkok, Thailand, Annual Progress Report, 1966).

To determine the prevalence and distribution of antigenic strains of R. tsutsugamushi in Thailand and to further evaluate the FA technique for analysis of R. tsutsugamushi infection in chiggers, a cooperative study was undertaken with the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. During the period of July to December 1977, chiggers were collected by the black plate method from various sites throughout Thailand (Figure 10), and shipped in vials of water to Kuala Lumpur for R. tsutsugamushi strain characterization and chigger species identification. Specimens from Pak Chong were collected from June through August 1978.

Table 15 summarizes the species of chiggers collected from the respective study sites. These included not only those chiggers examined for the presence of scrub typhus organisms, but also the ones which were dead upon arrival. L. (L.) deliense represents 84.5% of the total chiggers collected. L. (L.) miculum arvinum (3.6%) and L. (L.) fulleri (0.9%), both closely related to L. (L.) deliense, were also collected. Four unknown species, designated L. (L.) species A-D, have been found and are currently being identified.

Table 16 presents data on infection rates in chiggers examined from 4 of 7 study sites. Due to an excessive workload from on-going Malaysian and Thai studies, chiggers from 3 sites could not be examined. The presence of infected chiggers in these areas substantiated the findings of previous studies (15; 54; 55).

The number of infected chiggers by species is shown in Table 17. As expected, L. (L.) deliense comprised the majority (81.3%) of the infected chiggers. The other 3 infected Leptotrombidium (Leptotrombidium) species have not been previously recognized as vectors of scrub typhus; however, they are closely related taxonomically to L. (L.) deliense. The surprising finding is the single infected specimen of Microtrombicula chamlongi.

The results of typing the R. tsutsugamushi organisms in the infected chiggers are shown in Tables 18 and 19. There appears to



Figure 10. Locations of black-plated chigger collection sites in Thailand. (1) Chiang Mai, (2) Nakorn Ratchasima, (3) Ubon Ratchathani, (4) Surin, (5) Kanchanaburi, (6) Prachin Buri, (7) Pak Chong.

Table 15. Chiggers identified from Thailand during a study of Rickettsia tsutsugamushi

Location	<u>L. (L.) deliense</u>	<u>L. (L.) miculum arvinum</u>	<u>L. (L.) fulleri</u>	<u>L. (L.) species A</u>	<u>L. (L.) species B</u>	<u>L. (L.) species C</u>	<u>L. (L.) species D</u>	<u>L. (Trom.) paniculatum</u>	<u>Siseca rara</u>	<u>Walchiella oudemansi</u>	<u>Walchiella traubi</u>	<u>Microtrombicula chamlongi</u>	<u>Laurentella indica</u>	<u>Gahriepia species A</u>	Total
Chiang Mai	13	46	0	119	4	0	1	0	0	0	0	0	0	0	183
Nakorn Ratchasima	543	8	21	0	0	0	0	0	16	68	1	5	0	0	662
Ubon Ratchathani	949	0	0	0	0	0	0	0	0	0	0	1	0	0	950
Surin	145	0	0	0	0	15	0	0	0	0	0	0	0	2	162
Kanchanaburi	90	4	0	0	0	0	1	1	0	0	0	0	0	0	96
Prachin Buri	1	0	0	0	0	0	0	9	0	0	0	0	0	0	10
Pak Chong	162	23	0	0	0	1	0	1	0	0	0	0	1	0	188
Total	1903	81	21	119	4	16	2	11	16	68	1	6	1	2	2251

Table 16. Infection rates of chiggers examined for Rickettsia tsutsugamushi from Thailand.

Location	Total Chiggers Examined	Number Infected	Percent Infected
Chiang Mai	131	27	20.6
Nakorn Ratchasima	50	30	60.0
Ubon Ratchathani	579	26	4.5
Pak Chong	188	12	6.4
Total	948	95	10.0

A single chigger from one collection at Prachin Buri was also infected.

Table 17. Chigger species infected with Rickettsia tsutsugamushi from Thailand.

Location	<u>L. (L.) deliense</u>	<u>L. (L.) miculum arvinum</u>	<u>Microtrombicula chamlongi</u>	<u>L. (L.) species A</u>	<u>L. (L.) species B</u>	Total
Chiang Mai	13	5	0	7	2	27
Nakorn Ratchasima	29	0	1	0	0	30
Ubon Ratchathani	26	0	0	0	0	26
Prachin Buri	1	0	0	0	0	1
Pak Chong	9	3	0	0	0	12
Total	78	8	1	7	2	96

Table 18. Strains and strain combinations of Rickettsia tsutsugamushi from different geographical locations in Thailand.

	Chiang Mai	Nakorn Ratchasima	Ubon Ratchathani	Prachin Buri	Pak Chong	Total
Karp	5	1	8	0	10	24
Kato	0	1	0	0	1	2
TA686	1	1	3	0	1	6
TA716	7	0	6	0	0	13
TA763	9	7	1	0	0	17
Karp, TA716	1	2	1	0	0	4
Karp, TA763	1	3	0	0	0	4
Karp, TA716, TA763	1	2	0	1	0	4
Karp, TA686, TA716	0	0	2	0	0	2
TA716, TA763	2	12	0	0	0	14
TA686, TA716	0	0	4	0	0	4
TA686, TA763	0	0	1	0	0	1
Kato, TA716, TA763	0	1	0	0	0	1
Total	27	30	26	1	12	96

Table 19. Strains and strain combinations of *Rickettsia tsutsugamushi* by chigger species collected in Thailand.

	<u>L. (L.) deliense</u>	<u>L. (L.) species A</u>	<u>L. (L.) species B</u>	<u>L. (L.) miculum arvinum</u>	<u>Microtrombicula chamglongi</u>	Total
Karp	21	0	0	3	0	24
Kato	1	0	0	0	1	2
TA686	5	1	0	0	0	6
TA716	7	3	1	2	0	13
TA763	13	2	1	1	0	17
Karp, TA716	3	0	0	1	0	4
Karp, TA763	3	1	0	0	0	4
Karp, TA716, TA763	4	0	0	0	0	4
Karp, TA686, TA716	2	0	0	0	0	2
TA716, TA763	13	0	0	1	0	14
TA686, TA716	4	0	0	0	0	4
TA686, TA763	1	0	0	0	0	1
Kato, TA716, TA763	1	0	0	0	0	1
Total	78	7	2	8	1	96

be no correlation between either the strains and the collection sites or the strains and the chigger species. However, the findings clearly demonstrate the predominance of Karp and Karp-related (TA686, TA716, TA763) strains, a fact also observed in our studies in Malaysia (Table 14). Gilliam antigen was not observed in any case. Monotypic infections were seen in 64.6% of the infected chiggers; this again agrees with the findings in Malaysia.

FIELD STUDIES OF A SYSTEMIC ACARICIDE, DIMETHOATE, FOR THE CONTROL OF CHIGGERS (ACARINA: TROMBICULIDAE)

In a previous report (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977) this study was described in detail and preliminary findings were presented.

Because of the nature and habits of chiggers (trombiculid larvae), a single effective control measure is difficult to devise. As chiggers are often found in forested, swampy or grassy areas, many of the usual recommendations of insect control are not feasible. Many of the preferred habitats of chiggers are in vast forests, protected against the use of chemical treatment by dense vegetation. Control by removing the vegetation (i.e. burning, clearing and scraping), although effective, is usually limited to specific situations like camp grounds and construction sites.

The most common control measure is the use of chemical repellants, either by direct application to parts of the body or by clothing impregnation. This is most practical and effective when properly utilized.

In recent years, interest has increased in the use of systemic insecticides for the control of certain arthropods of medical and veterinary importance. Advantages of this means of control include: a relative ease of administering the chemical; a reduction in the amount of chemical applied; and the lack of environmental contamination by the chemical used. Systemic insecticides have proven effective against cattle grubs (14), fleas (7; 29; 30; 31; 32), lice (8), and the northern fowl mite (27).

To examine the feasibility of controlling chiggers through the use of a systemic insecticide, Dohany et al (11) administered orally 17 insecticides to guinea pigs. Of the insecticides tested, dimethoate was the only one which proved effective. In subsequent studies high chigger mortality occurred in guinea pigs and Hispid cotton rats given bait which contained as little as 0.1% dimethoate. The purpose of the current study was to determine the efficacy of dimethoate in controlling chiggers in a field situation.

A rodent bait of 0.2% technical grade dimethoate formulated with a 1:1 mixture of cracked corn and ground milo was maintained in wooden bait boxes. These bait stations measured 15 x 15 x 35 cm and were covered with a metal lid. Both ends were open to allow access to the bait. The bait was changed weekly with the uneaten portion being removed to determine the amount of bait consumed.

The bait was removed and rodents were trapped for 4 nights each month. The chiggers were collected from the rodents and the rodents were marked and released at the site of capture. The numbers and species of chiggers collected from each rodent were determined.

Two different study areas were examined during this field trial. The first area was comprised of approximately 2.7 acres of lalang grass (Imperata cylindrica) with a small area of scrub vegetation in its center. Forty-eight bait stations, located approximately 40 feet apart were placed in the study area. For the monthly trapping, 100 traps placed approximately 20 feet apart were used. A similar control site was established approximately 60 meters across a highway. The study in this habitat was terminated after 8 months when a fire eliminated most of the lalang in the dimethoate-treated area.

The second study site was located in an oil palm (Elaeis guineensis) scheme. The dimethoate-treated site consisted of approximately one acre of 6 year-old trees. A total of 100 bait stations were placed approximately 6 meters apart in a 60 x 60 meter grid. The same locations were used for the monthly trapping. A similar untreated area located approximately half a mile from the study site was trapped simultaneously.

Chigger foci were located prior to initiation of the study in the oil palm. Twenty-two foci were identified in the treated area and 15 in the control area. Monthly samples from each focus were taken with the black plate collection technique. Ten 10 x 12.5 cm black acrylic plates were used at each site. The chiggers were collected for identification.

Analysis of variance (ANOVA) was used to compare treated and control areas by the mean numbers of chiggers per rodent, the rodent entrapment rates and the mean number of chiggers per black plate over the whole period of the study. Trapping rates were standardized to the number of rodents per 100 traps per night.

Lalang Habitat

Figure 11 presents the average number of chiggers collected from Rattus tiomanicus, the predominant rodent trapped from both the treated and control areas within the lalang study site. The average number of chiggers per R. tiomanicus in the treated area was

less than that of the untreated throughout the entire time of the study. In general, the dimethoate-treated line follows the same pattern as that of the control, but at a reduced level. ANOVA shows the difference to be significant ($p < 0.01$). The differences were also significant at each of the sampling dates for which sampling was possible. There was also a significant difference ($p < 0.01$) between the control and treated areas for chigger rates at this species; as this was due to higher rates in the treated area, it does not reduce the significance of the difference in chigger carriage.

A similar picture was shown for chigger counts on *H. signatus* at the same sites. The control line was significantly lower than the treated area in the autumn ($p < 0.01$) and there was no significant difference in the autumn rates of the control and treated areas in either the difference between control and treated areas in either the first and second trapping one or two days.

As indicated during trapping by the control and treated areas having lower numbers of chiggers, the control area was also lower in the autumn ($p < 0.01$) and there was no significant difference between control and treated areas in either the first and second trapping one or two days.

The control area was also lower in the autumn ($p < 0.01$) and there was no significant difference between control and treated areas in either the first and second trapping one or two days.

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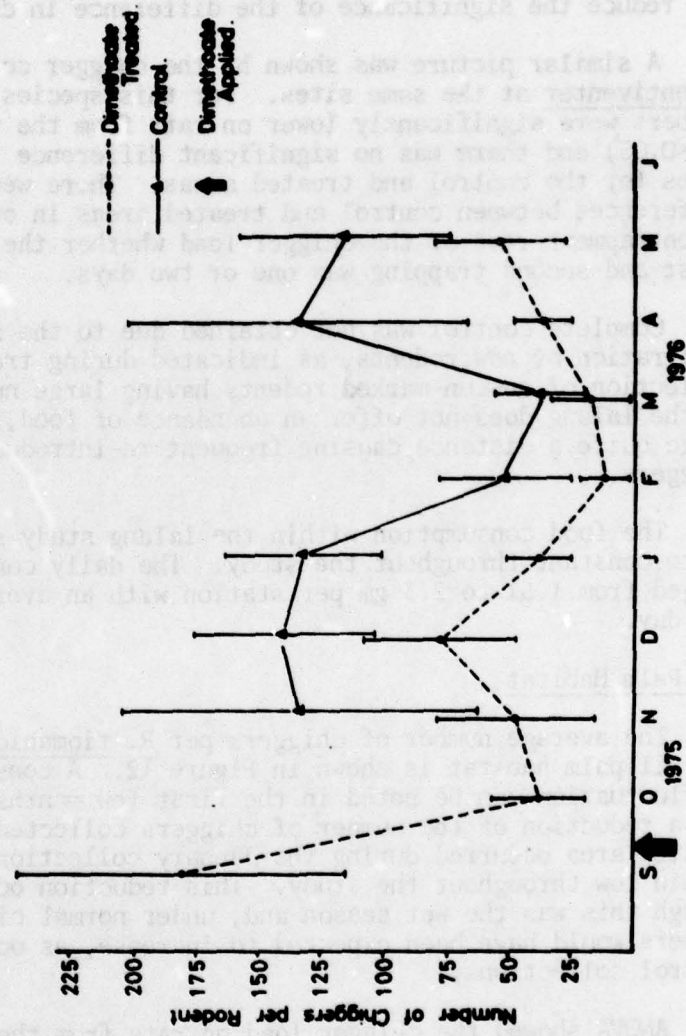


Figure 11. Average number of chiggers per rodent from dimethoate treated and untreated study sites in lalang.

less than that of the untreated throughout the entire time of the study. In general, the dimethoate-treated line follows the same pattern as that of the control, but at a reduced level. ANOVA shows the difference to be significant ($p < 0.01$). The differences were also significant of each of the sampling dates for which sampling was possible. There was also a significant difference ($p < 0.01$) between the control and treated areas for entrapment rates of this species; as this was due to higher rates in the treated area, it does not reduce the significance of the difference in chigger carriage.

A similar picture was shown by the chigger counts on R. argentiventer at the same sites. For this species the chigger numbers were significantly lower on rats from the treated area ($p < 0.05$) and there was no significant difference in the entrapment rates for the control and treated areas. There were no significant differences between control and treated areas in either the re-entrapment rate or the chigger load whether the interval between first and second trapping was one or two days.

Complete control was not obtained due to the constant immigration of new rodents, as indicated during trapping by the collection of new un-marked rodents having large numbers of chiggers. As the lalang does not offer an abundance of food, rodents often range quite a distance causing frequent re-introduction of new chiggers.

The food consumption within the lalang study sites remained quite constant throughout the study. The daily consumption of bait ranged from 1.51 to 2.3 gm per station with an average of 1.96 gm per day.

Oil Palm Habitat

The average number of chiggers per R. tiomanicus trapped from the oil palm habitat is shown in Figure 12. A considerable amount of fluctuation can be noted in the first few months of the trial, but a reduction of the number of chiggers collected from the treated area occurred during the January collection and continued to remain low throughout the study. This reduction occurred even though this was the wet season and, under normal circumstances, the numbers would have been expected to increase, as occurred in the control collections.

ANOVA showed the chigger load on rats from the treated area to be significantly lower ($p < 0.01$) than on those from the control area. The differences were also significant on 7 of the 12 sampling dates on which comparison was possible.

Figure 13 compares the average number of chiggers collected per black plate within the treated and untreated oil palm areas. During the initial 4 months of the study, the number of chiggers per

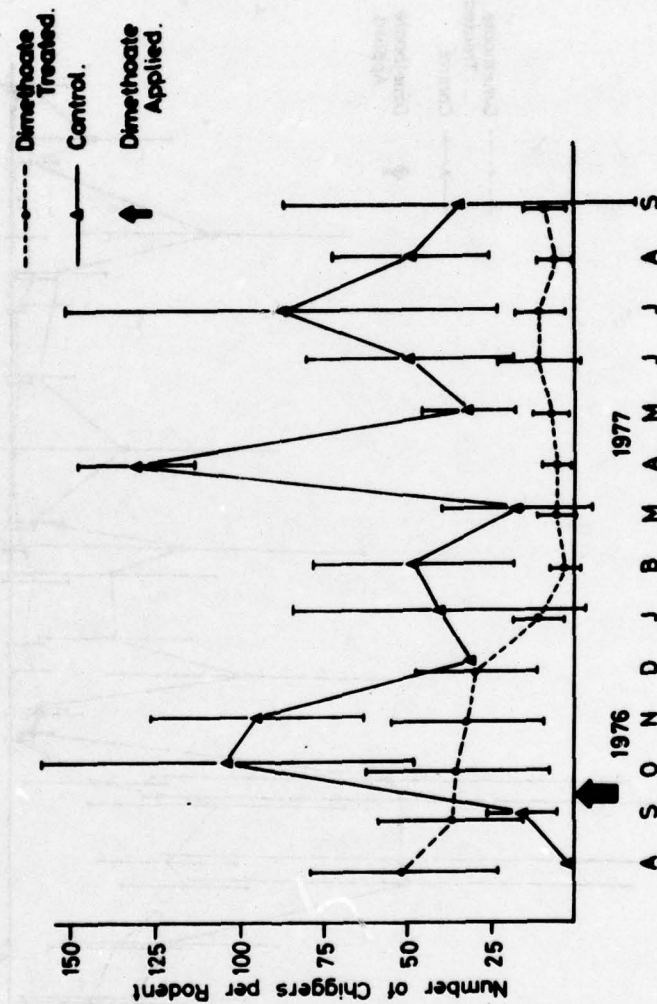


Figure 12. Average number of chiggers per rodent from dimethoate treated and untreated study sites in oil palm.

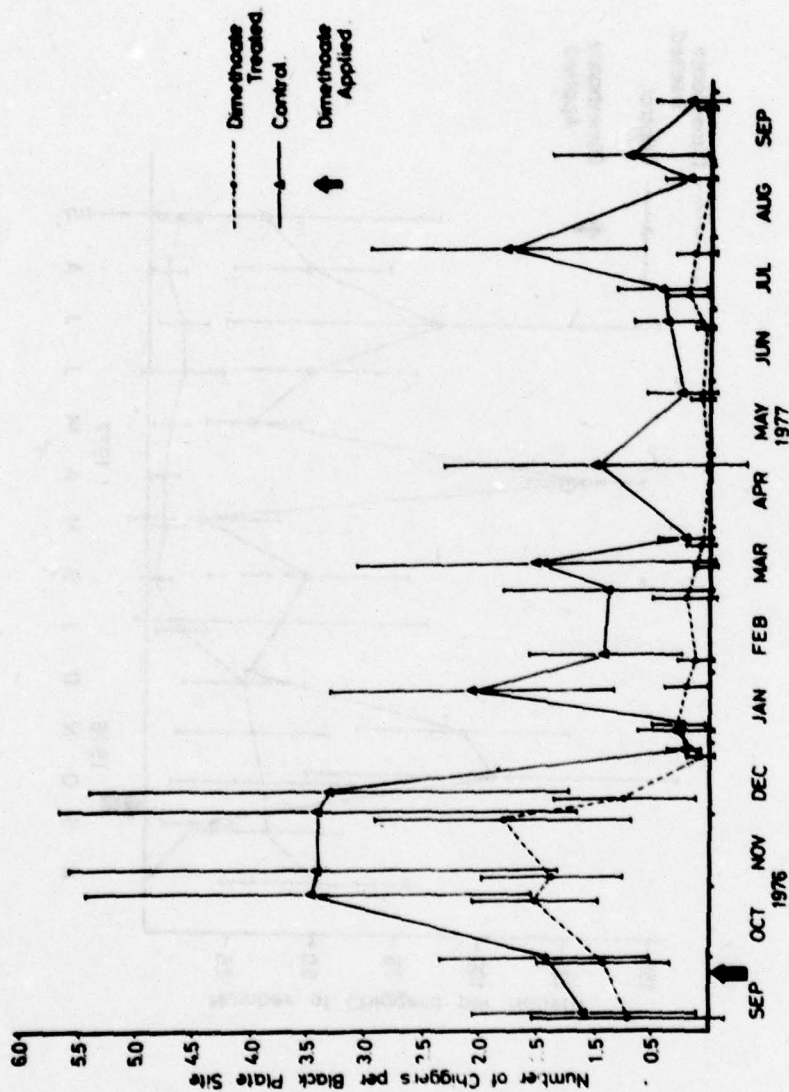


Figure 13. Average number of chiggers per black plate site from dimethoate treated and untreated study sites in oil palm.

black plate from the treated area tended to follow a similar pattern to that of the untreated area. The number of chiggers per black plate dropped considerably after December, with the level approaching zero during much of the remaining time.

ANOVA showed that the black plate yield from chigger foci in the treated area to be significantly lower ($p < 0.01$) than the yield from those in the control area, despite the environmental effects of the chigger population of both areas.

Although a large food source was available in the form of oil palm fruit, an equally good acceptance of bait in comparison to the lalang study site was noted. The daily consumption per station ranged from 1.23 gm to 5.22 gm, with an average of 2.56 gm.

TROMBICULID MITE COLONIES

Two colonies of infected vector chiggers are continuing to be maintained by the Department of Acarology. The infected colony of L. (L.) fletcheri, currently in its 27th generation, was collected from a lalang field near Kampung Jenderam, Peninsular Malaysia. The L. (L.) arenicola infected colony, in its 14th generation, was collected under vegetation along a sandy beach near Mersing, Peninsular Malaysia.

The transovarial and transtadial infections of both colonies have been studied in detail (38; 45). Additionally, these colonies along with uninfected colonies of the same species have been used in transmission studies and studies of their life cycles (13; 40; 60).

Characterization of R. tsutsugamushi organisms existing in 22 lines of the 25th generation of the L. (L.) fletcheri colony and 23 lines of the 13th generation of the L. (L.) arenicola colony has been initiated. (See Characterization of Strains of R. tsutsugamushi Present in Infected Laboratory-Reared L. (L.) arenicola and L. (L.) fletcheri Chiggers). Additionally, both the infected and uninfected colonies have been used to supply specimens to WRAIR for electron microscopy studies and vaccine testing. During the past reporting period over 200 adults have been reared, tested for R. tsutsugamushi infectivity and shipped to WRAIR.

PERSISTENCE AND REACTIVATION OF R. TSUTSUGAMUSHI INFECTIONS
IN LABORATORY MICE

Previous studies on rodents suggest that persistent or dormant R. tsutsugamushi infections may be common (17). In endemic areas, a large portion of wild rodents have yielded isolates from blood and/or tissue pools. Occurrence of R. tsutsugamushi organisms in lymph nodes of a patient infected 1-2 years before was described by Smadel et al. (49).

This study was designed to determine the existence of dormant infections in mice experimentally infected with R. tsutsugamushi and the reactivation of such infections by inoculating the mice with a heterologous strain of R. tsutsugamushi; by infecting the animals with a non-rickettsial organism, Plasmodium berghei; and by introducing an immunosuppressant.

A large number of ICR mice were infected with the avirulent strain of TA678. Several tests were performed to insure that the mice were infected solely with the TA678 strain. A group of glycogen-treated (45) mice were infected with the inoculum, and thrice weekly for 4 weeks, peritoneal exudates from 3 mice were collected and examined for the presence of rickettsial organisms using the fluorescent antibody (FA) technique. Peritoneal cells prepared from infected mice 4 to 23 days post-inoculation contained rickettsiae which reacted only with anti-TA678 serum. In addition, 10 normal mice were infected with the inoculum, and after 28 days, were challenged with a lethal dose (10^4 MLD₅₀) of the Karp strain. All animals survived the challenge. Another 10 normal mice were given the inoculum, and they were bled individually from the retroorbital plexus on a weekly basis to measure the presence of antibody. Only TA678 antibodies were detected post-inoculation. The titration of the inoculum in mice showed MLD₅₀ of $<10^{-1.5}$ and MID₅₀ of $10^{-6.4}$.

Ten randomly selected, infected mice were serially bled on a monthly basis to record their antibody titers. The maximum titers of 1/25 were reached on day 14 post-inoculation, and after day 90, the titers were at 1/5. After 8½ months post-infection when the antibody titers had fallen below 1/5, 10 mice were bled, and the blood from each mouse was inoculated into normal mice to determine the presence of rickettsemia (5). Suspensions of liver, spleen, kidney, and lymph nodes from the same animals were inoculated into other normal mice to detect the presence of persistent organisms (5). All blood samples were negative, whereas 4/10 tissue pools were positive for rickettsiae. At 19 months post-infection, 10 additional mice were examined. Although none of the mice were rickettsemic, 6/10 tissue pools contained rickettsiae.

The remaining mice, infected with TA678 19 months previously, were divided into 3 large groups. One group was inoculated with the

heterologous TA586 strain, a second with P. berghei, and the third group was given cyclophosphamide. Thrice weekly for 3 weeks, 4 mice were bled and each of the 4 samples was inoculated into 3 glycogen-treated mice. When sick or on day 14, mice were harvested. The peritoneal fluids from the 3 mice were pooled and processed for examination by the FA technique. A suspension prepared from blood, liver and spleen from selected mice was inoculated immediately into other glycogen-treated mice (2nd passage). Blood and tissues from other mice were stored at -70°C. The peritoneal fluids from the 2nd passage mice were also pooled when the rodents became ill or on day 14 and processed for examination by the FA technique. The results are shown in Table 20. In the group of mice inoculated with the TC586 strain, minimal reactivation of TA678 organisms was seen only after the 2nd passage. However, cyclophosphamide reactivated TA678 infection in most of the mice tested, and all rickettsemias were apparent in the first mouse passage. There were no organisms detected in peritoneal fluids from mice given P. berghei; however, the infectivity of the inoculum was sufficiently high that no mice survived beyond the 11th day.

This study demonstrated the persistent nature of the rickettsiae, being present in tissues 19 months post-infection. In addition, reactivation of the dormant organisms occurred by subjecting the infected mice to subsequent heterologous infection or to treatment with an immunosuppressant.

EARLY DETECTION OF RICKETTSIA TSUTSUGAMUSHI IN PERIPHERAL MONOCYTE CULTURES DERIVED FROM EXPERIMENTALLY INFECTED MONKEYS AND DOGS

In the previous report (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977), the use of the monocyte culture technique for the isolation of R. tsutsugamushi from experimentally infected monkeys and dogs was described. In this first series of studies, cultures were examined on days 9 through 16 only. During the past year, a second series of studies was completed wherein cultures were examined after 2 days.

Two coverslips were removed on different days, ranging from 2 to 16 days of culture. One coverslip was heat-fixed and stained with the Giemsa technique, while the other was fixed in acetone for 10 min and stained with fluorescein conjugated anti-R. tsutsugamushi (Karp strain) rabbit serum (23). The test was controlled by using both conjugated normal rabbit serum and monocyte cultures prepared from uninfected control animals.

To determine the infectivity of the original blood samples and the fluid components in the monocyte cultures, 0.2 ml of each

Table 20. Reactivation of TA678 rickettsiae in mice by inoculation with heterologous TC586 strain, inoculation with Plasmodium berghei, and treatment with cyclophosphamide.

Days Post-Inoculation/ Treatment	TC586		<u>P. berghei</u>	Cyclophosphamide
	TA678	TC586	TA678	TA678
2	2/4*	1/4	0/4	4/4
4	0/4	4/4	0/4	4/4
6	0/4	4/4	0/4	2/4
9	1/4	4/4	0/4	2/4
11	0/4	4/4	0/3	2/4
13	0/4	4/4	-**	1/4
16	0/4	4/4	-	3/4
18	0/4	4/4	-	3/4
20	0/4	4/4	-	0/4

* Number of specimens showing positive fluorescence/total number of specimens observed.

**Not done.

specimen was inoculated intraperitoneally into laboratory mice (5).

Sera were collected at each bleeding and tested by indirect immunofluorescent technique to determine the homologous antibody titers (41).

Rickettsial organisms were identifiable by direct fluorescent antibody staining of peripheral monocyte cultures prepared from all infected animals (Figure 14). Although organisms could sometimes be observed in cultures stained after only 2 days incubation, rickettsiae were nearly always found in cultures incubated 7 days or longer. Monocytes derived from cynomolgus monkeys yielded the most consistent results, with the organisms being observed in cultures derived from animals through day 23 post-infection. Dogs presented a more sporadic picture, but the rickettsiae were seen throughout the same time span. The cultures from silvered leaf monkeys contained fewer intact cells and more extracellular organisms, especially during the peak of infection. Rickettsiae could be isolated from the media of cultures containing cells with identifiable organisms, and the isolation of R. tsutsugamushi in monocyte cultures derived from infected animals corresponded with isolations from the blood of the same animals using the mouse isolation technique.

Simultaneous observations of Giemsa-stained cultures demonstrated rickettsial-like organisms; however, the organisms were easily discernable only during the height of infection. At other times when the rickettsiae were present in decreased number, examination of Giemsa-stained cultures proved more difficult in that the organisms were often difficult to differentiate from other cellular components. Therefore, the use of direct immunofluorescence was the much preferred technique for detecting the rickettsial organisms. The rickettsiae did not stain with normal conjugate. Uninfected monocyte cultures did not show any organisms with either immunofluorescent or Giemsa staining.

Specific antibodies to Karp strain developed in all animals between days 13-20 post-inoculation.

The standard methods for isolating rickettsial organisms are long and laborious, often requiring a ready supply of various laboratory animals. The use of monocyte cultures derived from the infected host as a means of isolating rickettsiae offers several advantages over other standard methods. The time required for isolation is markedly reduced. The technique is simple and inexpensive in that the need for maintaining laboratory animals, tissue cultures and embryonated eggs is eliminated. The organisms are usually recovered in pure culture without having been grown in cells other than those of the original host. The latter is an important consideration in the isolation of organisms which may serve as potential vaccines.



Figure 14. Fluorescent antibody staining of *R. tsutsugamushi* organisms in the cytoplasm of monocytes cultured from an infected monkey (x 2500).

The success in detecting R. tsutsugamushi organisms in peripheral monocyte cultures derived from experimentally infected animals has prompted us to apply this methodology to the isolation of R. tsutsugamushi from human scrub typhus patients. Currently a study is in progress to evaluate the efficiency and sensitivity of this culture technique.

CLINICAL RESPONSES OF SILVERED LEAF MONKEYS TO INFECTION WITH SELECTED STRAINS OF RICKETTSIA TSUTSUGAMUSHI

Extensive studies of Rickettsia tsutsugamushi isolates recovered from human beings, mites and small mammals throughout the scrub typhus endemic area have clearly demonstrated the antigenic diversity of the organism.

In addition to antigenic variation, strains of R. tsutsugamushi can differ in their virulence for various laboratory animals. Robinson et al. (42) experimentally infected silvered leaf monkeys with 5 strains selected on the basis of varying virulence in mice. Their data showed that strains avirulent for mice produce little or no disease in monkeys, whereas monkeys inoculated with mouse virulent strains developed signs of disease compatible with the human infection.

The 5 strains examined in the above study represented a portion of 9 strains currently used in this laboratory for diagnostic and epidemiological studies. The study described here was undertaken to examine the response of silvered leaf monkeys to the 4 remaining strains and was designed to allow for direct comparison with the study reported by Robinson et al. (42).

The silvered leaf monkeys (Presbytis cristatus) were purchased from Malaysian Livestock Exporters (Petaling Jaya, Selangor, Malaysia). The monkeys were maintained as recommended by Walker et al. (58).

Outbred ICR mice, 16 to 20g, were obtained from the Division of Laboratory Animal Resources of the Institute for Medical Research.

The 4 strains used were TC586, TA716, TA763, and TH1817, all of which were isolated in Thailand (42). The seed materials were prepared in embryonated hens' eggs (SPAFAS, Norwich, Conn.) and stored at -196C. For inoculation, the stock solutions were diluted to the desired concentration in Snyder I diluent (2). The 2 doses given were approximately 10^6 and 10^4 mouse infectious dose (MID₅₀). Each animal was inoculated intradermally with 0.05 ml on the medial surface of the left thigh.

The animals were observed daily for clinical manifestations, and the venous blood was collected at selected times to determine the white blood cell (WBC), red blood cell (RBC), and differential counts, and packed cell volumes.

Virulence in Mice: The seed suspensions of the 4 strains were titrated in mice concurrently with the inoculations in monkeys. The virulence of the TA763 strain was similar to that seen with the Karp strain, being highly lethal for mice. On the other hand, TA716 strain was rarely lethal but immunized the animals with no signs of disease. The TC586 and TH1817 strains were of intermediate virulence between the above 2 strains.

Virulence in Monkeys: Responses in silvered leaf monkeys are summarized in Table 21. Febrile responses ($\geq 103.2^{\circ}\text{F}$) were observed in only 2 monkeys: for 1 day in an animal receiving $10^{6.1}$ MID₅₀ of TA716; and for 5 days in a monkey given $10^{6.7}$ MID₅₀ of TC586. Regional lymphadenopathy was noted in animals receiving the higher doses of the strains, but no eschars developed in any animals. One monkey given $10^{6.5}$ of MID₅₀ of TH1817 died 26 days post-inoculation, but death could not be definitively associated with the infection.

Hematology Results: In all cases, leukopenia developed between days 9 and 16 post-inoculation. Otherwise, the results were unremarkable when compared with the control animals.

In an earlier study Robinson et al. (42) observed that the severity of disease in silvered leaf monkeys experimentally infected with various strains of *R. tsutsugamushi* was related to the virulence of the strains in mice. In monkeys inoculated with mouse virulent strains, clinical and hematological signs were evident, whereas the monkeys inoculated with mouse avirulent strains few, if any, signs were noted. In the current study, using different strains, a correlation between virulence in mice and virulence in silvered leaf monkeys was not observed. TA763 strain, for instance, is similar to the Karp strain, in that both are serologically related and both are virulent for mice. However, Karp is virulent in monkeys, TA763 is not. TA716 and Karp strains are serologically related, but TA716 has very low virulence for both mice and monkeys. In addition, TC586 and Gilliam strains are serologically indistinguishable and have similar virulence properties in mice. Nevertheless, TC586 does not produce the expected disease that Gilliam does in monkeys. Recently, Groves et al. (18) have demonstrated the susceptibility and the resistance of different strains of both outbred and inbred mice to the same strain of *R. tsutsugamushi*. This finding substantiates the fact that extrapolation of data derived from one animal cannot be made to another animal, or even within the same animal species.

Table 21. Responses of silvered leaf monkeys to ID inoculation of selected strains of Rickettsia tsutsugamushi

Strain, Dose*	No. with Fever (Day)**	No. with Lymphadenopathy	No. with Eschar	No. with Rickettsemia (Day)***
TC586				
6.7	1 (19-22,25)	2	0	2 (4-28)
4.7	0	2	0	2 (11-28)
TA716				
6.1	1 (16)	1	0	1 (4-28)
2.3	0	0	0	2 (6,12-28; 12-28)
TA763				
6.2	0	2	0	2 (2-11; 2-25)
4.2	0	0	0	2 (7-23; 9-21)
TH1817				
6.5	0	1	0	2 (7-28)
4.5	0	2	0	2 (9-28)

Note: Two monkeys were inoculated with each dose. One monkey in TA716 6.1 MID₅₀ group died on day 3.

* Log₁₀ mouse infectious doses by the IP route.

** Days post-inoculation on which fever occurred.

*** Days post-inoculation on which rickettsemia was demonstrated.

EXPERIMENTAL R. TSUTSUGAMUSHI INFECTIONS IN MONKEYS

The susceptibility of monkeys of scrub typhus organisms has been reported previously (20; 25; 26). Silvered leaf monkeys have been used in this unit for experimental infections with different R. tsutsugamushi strains (42; 59), but results of some studies (59) are difficult to interpret due to simultaneous inoculations with multiple strains.

Experimental infections induced by intradermal (ID) inoculations of both silvered leaf and cynomolgus monkeys with Karp, Gilliam, and Kato strains have been reported (Walter Reed Army Institute of Research Annual Progress Report, 1 October 1976 - 30 September 1977). The Gilliam strain was the most virulent of the 3 strains in both species of monkeys. Generally, both monkeys reacted similarly, but there were 2 significant differences: (1) death occurred in Gilliam-infected silvered leaf monkeys, while there were none in cynomolgus monkeys; and (2) the antibody responses in cynomolgus monkeys were greater than in silvered leaf monkeys.

A limited study was performed to determine the effect of infecting the 2 species of monkeys intravenously (IV) with mouse virulent (10^6 MLD₅₀ of Karp strain) and mouse avirulent (10^6 MID₅₀ of TA686 strain) strains. All monkeys infected with the Karp strain showed systemic signs before dying. In the case of TA686 infections, clinical manifestations were less evident than in Karp infections, and the only deaths which occurred were in 2 of 3 silvered leaf monkeys.

Surviving silvered leaf monkeys from various studies were reinfected ID with $10^{5.4}$ MID₅₀ of the Gilliam strain, and their responses were noted. The silvered leaf monkeys included those infected 10 months previously with Karp, Gilliam, and Kato strains; those infected 7 months earlier with TC586 and TH1817 strains; and those infected 4 months previously with TA686 and TA716 strains. Unfortunately, the numbers were small (14). Three monkeys infected 4 months previously and one 7 months earlier had low but existing antibody titers to their respective infecting strains at the time of challenge. The monkeys infected previously with TC586 demonstrated regional lymphadenopathy only. TC586 strain is serologically related to the Gilliam strain. In contrast, those previously infected with TH1817 developed fever, lymphadenopathy, eschar, and 2 of 3 animals died. In other animals infected 4 and 10 months previously, the responses varied and could not be correlated with either prior infecting strain or presence of antibodies.

Greater numbers of cynomolgus monkeys were available for the reinfection study. The animals had been infected 5 to 22 months earlier with one of the following strains: Gilliam, Karp, Kato or TA686. All monkeys infected 5 to 7½ months prior to reinfection

as well as those infected previously with the Gilliam strain, regardless of the duration between primary infection and reinfection, possessed low antibody titers. In this study, animals were divided into 2 groups, one being challenged ID and the other IV with $10^{5.5}$ MID₅₀ of the Gilliam strain. All ID-challenged animals demonstrated regional lymphadenopathy and eschar formation without any correlation to the presence of antibody or to the strain comprising the initial infection. Those challenged IV showed varied responses, with most developing either fever and/or lymphadenopathy. There were no deaths among the challenged animals.

In both species of monkeys previously infected with either Gilliam or TC586 strain, only homologous antibody responses were evident upon challenge with the Gilliam strain. However, in monkeys previously infected with the other strains, reinfection with the Gilliam strain elicited a heterologous response. In these animals, antibodies to Karp, Kato, TA686, TA716, and TA763 as well as Gilliam were demonstrated.

BREEDING CYNOMOLGUS MONKEYS IN CAPTIVITY FOR USE IN SCRUB TYPHUS RESEARCH: A FEASIBILITY STUDY

Background: An important segment of the scrub typhus research program is the development of an intermediate animal model for the disease. To this end 2 species of primates, the silvered leaf monkey (Presbytis cristatus) and the cynomolgus monkey (Macaca fascicularis), have been studied. Based on acquisition, maintenance in the laboratory, and response to infection, young cynomolgus monkeys are the most acceptable for large scale studies.

Since many adult, wild-caught cynomolgus monkeys have antibody to Rickettsia tsutsugamushi (59), it is apparent that this animal is frequently exposed in its natural habitat. To establish a baseline for evaluation of the cynomolgus monkey as a model for scrub typhus and to define parameters for selection of wild caught animals for laboratory studies, the requirement for colony-reared animals, free from exposure to R. tsutsugamushi, became essential. To meet this requirement and to evaluate the feasibility of breeding cynomolgus monkeys in captivity, a small breeding colony of cynomolgus monkeys was established.

Materials and Methods: The colony consisted of 2 groups of monkeys each containing 10 females and 1 male. Group Number 1 was established in September 1975 and group Number 2 was established in February 1976. The animals were all wild caught and were subjected to a 6 month period of conditioning and compatibility testing prior to final grouping. During the conditioning period each monkey was

wormed twice with Levamisole (Nemicide, Imperial Chemicals Industries Ltd) and received 5 tuberculin tests. Subsequent to establishment of the colony, the monkeys were tuberculin tested every 6 months. A commercial monkey chow was provided ad libitum.

The breeding building was constructed of wood support posts with walls of 2" x 2", 14 gauge wire mesh and zinc sheet roofing. The floor plan provided 4 separate rooms (2 on each side of the building) 8 feet wide, 15 feet long and 7 feet high. There was a door separating the rooms on each side which could be permanently opened to provide a space of 8 feet wide by 30 feet long. In addition, there was a working area which was 9 feet by 22 feet which also served to house individual cages when necessary. The floor was a poured concrete slab.

Three different caging systems for nursing mothers and their offspring were evaluated. One method was to allow the nursing mother and baby to remain in the breeding room with the rest of the group and remove the baby once it had been weaned. Another technique was to place the female and nursing infant in a separate wire mesh cage (approx. 2' x 2' x 2') within the breeding room shortly after parturition. This technique enabled the mother to maintain some association with her original breeding group but prevented fighting between the mother and other members of the group. A third system which was used toward the end of the nursing period was to place the mother-infant pair in a 2 part cage which allowed the infant to crawl through a small opening and obtain food undisturbed by the mother. Once the infant was completely weaned it was removed and the mother was returned to the breeding group.

Complete records were maintained on all of the animals which included dates infants were born, weaning dates, tuberculin tests, complete medical histories and necropsy reports. In addition, all of the offspring were weighed once a month, tooth eruption patterns were recorded and blood was drawn for evaluation which included determinations/or hematocrit, hemoglobin, WBC, RBC, thrombocyte count, differential count and erythrocyte sedimentation rate.

The colony has remained in continuous operation since its inception in 1975 except for a period of 4 months from December 1976 to April 1977 when all of the animals were housed in separate cages which was necessitated by a requirement to change the location of the colony.

Production: From the inception of the project in September 1975 through September 1978, the total production for group No.1 has been 22 babies (13 males and 9 females). Of the 22 offspring 1 was stillborn, 5 died during the nursing period, 9 are still nursing and 7 have been weaned. The average time required for weaning was 248 days with an average weight at weaning of 1.028 kg. for females and

.837 kg for males. Of the 10 breeder females, 4 have produced 3 offspring each, 5 of the females have produced 2 offspring each and one of the females has remained barren throughout the project. The average time from availability for mating until parturition for these 22 births was 198 days. Counting a 171 day pregnancy (21) the average time in the colony for conception to take place was approximately 27 days. The compatibility of the breeder animals has remained generally good throughout the project and no animals have been replaced.

Group 2 which was started in February of 1976 has produced a total of 15 babies (10 males and 5 females). Of this total 1 was stillborn, 4 died during the nursing period, 4 are still nursing and 6 have been weaned. The average time required for weaning was 231 days, with an average weight at weaning of .877 kg. for males and .810 kg. for females. One of the breeder females has produced 3 offspring, 3 have produced 2 offspring each and 6 have produced one offspring each. The average time from availability for mating to parturition for these 15 births was 238 days with an average of 67 days required time for conception as described in the previous group. This group experienced considerable compatibility problems when it was first formed. The male and 7 of the females were replaced during the first year of breeding. As a result of these problems only 3 offspring were produced prior to the 4 month period when the animals were housed individually. When the colony was reunited in April of 1977 the group compatibility was similar to that described for group Number 1 and the remainder of the offspring of group Number 2 were produced after April of 1977.

Weaning Techniques: Allowing the nursing mother and baby to remain in the breeding room with the rest of the group was the least time consuming technique for maintenance personnel; however, this resulted in considerably more fighting in the group as a whole and injuries were often sustained by the newborn infant. Four deaths resulted from trauma inflicted on the newborn. As a result of this experience this technique was abandoned and none of the offspring were weaned by this method.

To eliminate the fighting problem the second system was initiated which utilized an individual cage inside of the breeding room. Sixteen animals were weaned using this method and the average time required for weaning was 242 days. This system eliminated much of the fighting and no infant deaths were attributed to trauma while it was employed. The third system utilizing the two part cage which allows the infant access to food without the interference of the mother was initiated because it was noted that often the mother became a hindrance to the weaning process and would not allow the infant to eat even though it showed interest in trying to eat on its own. This system was used after the mother/infant pair had been housed in the breeding room in an individual cage. Five animals have been weaned using this technique and the average time for weaning was 215 days.

Weight Gains: The mean and standard deviation for weight recorded once per month is summarized in Table 22.

Tooth Development: The mean and standard deviation in days for deciduous tooth eruption is given in Table 23. Information is given separately for males and females. Since dentition was observed only once per month these figures can only be considered accurate to within 30 days.

The mean and standard deviation in days for the permanent dentition observed up to September 1978 is given in Table 24. Information format is the same as that for the deciduous dentition.

Hematology: Hematological data is still under evaluation and will be reported at a later date.

THE USE OF TETRACYCLINE TO CONTROL CANINE EHRLICHIOSIS

Canine ehrlichiosis, which is caused by the rickettsial organism, Ehrlichia canis, has been shown to be a devastating disease among police and military dogs, particularly in Southeast Asia (9; 50; 61; 62).

Amyx et al. (1) showed that tetracycline given at the rate of 30 mg/lb/day will cause remission of the signs of ehrlichiosis in dogs treated during the early stages of the disease, but in these studies 2 of 15 dogs so treated remained carriers. These same workers found that low levels of tetracycline (3 mg/lb/day) rendered dogs refractory to infection with Ehrlichia canis (1). Military dogs placed on daily prophylactic doses (3 mg/lb/day) of tetracycline may be expected to perform their duties in an endemic area without developing the disease (10; 63); however, during sustained training and operations, or under numerous other conceivable circumstances, the daily administration of tetracycline may be interrupted.

In the absence of available diagnostic support, personnel responsible for the care of military dogs may feel impelled to therapeutically treat every dog where there is suspicion that prophylaxis may have been interrupted. This has a number of disadvantages: (1) although the cost of tetracycline may not always be significant, the cost of the drug has interfered with its use in military dog units of a number of allied forces; (2) in an active military dog unit some dogs may be on therapeutic levels of tetracycline almost continuously; (3) the maintenance of records may become impossible in an active unit; (4) some dogs would undoubtedly be required to go on training or operational exercises while on therapeutic tetracycline, and suspected breaks in

Table 22. Average weight of cynomolgus monkeys born and raised in captivity.

Age (months)	Sex	Mean (N) *	Standard Deviation	Age (months)	Sex	Mean (N)	Standard Deviation
0-1	M	.315 (22)	.056	12-14	M	1.175 (11)	.096
	F	.327 (13)	.055		F	1.150 (8)	.083
1-2	M	.394 (21)	.073	14-16	M	1.194 (11)	.145
	F	.390 (12)	.066		F	1.212 (8)	.108
2-3	M	.467 (19)	.084	16-18	M	1.271 (11)	.157
	F	.496 (12)	.100		F	1.300 (8)	.095
3-4	M	.532 (17)	.082	18-20	M	1.405 (9)	.104
	F	.611 (9)	.118		F	1.389 (7)	.162
4-5	M	.612 (17)	.101	20-22	M	1.468 (9)	.111
	F	.663 (10)	.120		F	1.426 (8)	.185
5-6	M	.659 (16)	.082	22-24	M	1.503 (6)	.093
	F	.759 (8)	.142		F	1.531 (9)	.205
6-7	M	.710 (14)	.102	24-26	M	1.598 (6)	.123
	F	.818 (10)	.132		F	1.741 (7)	.130
7-8	M	.799 (13)	.104	26-28	M	1.701 (3)	.104
	F	.839 (6)	.133		F	1.717 (8)	.133
8-9	M	.869 (9)	.087	28-30	F	1.848 (5)	.172
	F	1.027 (5)	.097				
9-10	M	.921 (4)	.156	30-32	F	1.911 (5)	.165
	F	1.023 (7)	.083				
10-11	M	.968 (9)	.148	32-34	F	1.939 (3)	.147
	F	1.083 (5)	.082				
11-12	M	1.046 (6)	.136				
	F	1.130 (5)	.043				

* Weight in kg. (Number of animals observed).

Table 23. Age when deciduous dentition was noted.

	Males		Females	
	Mean (N) *	Standard Deviation	Mean (N)	Standard Deviation
I ₁ maxilla	33 (20)	17	27 (13)	11
mandible	33 (20)	17	27 (14)	11
I ₂ maxilla	49 (20)	17	42 (11)	13
mandible	42 (20)	19	39 (11)	12
C maxilla	92 (20)	20	78 (11)	13
mandible	92 (20)	20	76 (11)	17
M ₁ maxilla	97 (20)	18	78 (11)	13
mandible	97 (20)	20	78 (11)	13
M ₂ maxilla	178 (14)	30	153 (10)	23
mandible	174 (15)	24	150 (10)	24

*Age in days (number of animals observed)

Table 23. Age when deciduous dentition was noted.

	Males		Females	
	Mean (N) *	Standard Deviation	Mean (N)	Standard Deviation
I ₁ maxilla	33 (20)	17	27 (13)	11
mandible	33 (20)	17	27 (14)	11
I ₂ maxilla	49 (20)	17	42 (11)	13
mandible	42 (20)	19	39 (11)	12
C maxilla	92 (20)	20	78 (11)	13
mandible	92 (20)	20	76 (11)	17
M ₁ maxilla	97 (20)	18	78 (11)	13
mandible	97 (20)	20	78 (11)	13
M ₂ maxilla	178 (14)	30	153 (10)	23
mandible	174 (15)	24	150 (10)	24

*Age in days (number of animals observed)

Table 24. Age when permanent dentition was first noted.

	Males		Females	
	Mean (N) *	Standard Deviation	Mean (N)	Standard Deviation
M ₁ maxilla	579 (6)	36	520 (4)	18
mandible	503 (6)	30	483 (4)	41
I ₁ maxilla	816 (1)	-	900 (3)	51
mandible	816 (1)	-	895 (4)	47
I ₂ maxilla			940 (1)	-
mandible			940 (1)	-

* Age in days (number of animals observed).

administration would further complicate the problem; (5) the frequent therapeutic regimes may lead to side reactions which would interfere with the effectiveness of the dog.

Numerous questions arise when considering the consequences that might result when prophylactic tetracycline (3 mg/lb/day) is administered to dogs in various stages of the disease:

1. Will low level tetracycline eliminate rickettsemia in an infected dog?
2. Will low level tetracycline eliminate infections from carrier dogs?
3. What is the effect of such treatment on antibody levels revealed by the IFA test if: (a) the organism is eliminated, (b) the organism is not eliminated?
4. Would dogs which had been carriers and subsequently were freed from infection by chemotherapy, prove to be immune to reinfection?
5. Is this protective immunity characterized by (a) rejection of the infection, (b) infection in the absence of hematologic and clinical signs of the disease, (c) infection with hematologic abnormalities in the absence of clinical signs of the disease, (d) infection with hematologic and clinical signs of the disease?
6. What is the pattern of antibody responses in each possible situation cited in 1-5?

To answer these questions a series of studies was undertaken wherein prophylactic tetracycline (3 mg/lb/day) was initiated during the incubation period (3 & 7 days post-inoculation), when early signs of disease had developed (14 days PI), and during chronic infection (60 days PI). The isolate used in the studies was recovered from a dog in Negri Sembilan, Peninsular Malaysia (33). Prophylactic tetracycline was administered for 30 days. To determine rickettsemia, blood was collected from each dog on the day of initiation of treatment, during treatment, and following treatment, and was inoculated into a susceptible dog. After chemotherapy was terminated the dogs were observed for signs of disease and were reinfected to determine the degree of resistance to infection or disease. Serum specimens were collected weekly from all dogs in the study and examined by the IFA test for *E. canis* (39). The serological tests were performed by Dr. Miodrag Ristic, College of Veterinary Medicine, University of Illinois.

Although a large number of serum specimens remain untested and data is yet to be analyzed, some generalizations can be made regarding the findings: (1) 30 days of low level tetracycline

cleared all dogs of infection regardless of the time tetracycline was initiated; (2) signs of disease in dogs started on tetracycline 14 days post-inoculation disappeared rapidly after treatment was initiated; (3) all dogs treated 3, 7 and 14 days post-inoculation, and cleared of the infection, were fully susceptible to infection and disease following reinfection, indicating that no immunity had developed; (4) dogs treated 60 days post-inoculation and cleared of the infection were susceptible to reinfection but showed little or no clinical signs, indicating that some immunity to disease had developed; (5) transitory antibody responses were noted in all dogs regardless of when tetracycline therapy was instituted; (6) all dogs which were reinfected after tetracycline treatment was discontinued developed high antibody titers to E. canis.

AUTOMATIC DATA PROCESSING (ADP) SYSTEM

From studies initiated in early 1975 on scrub typhus and other fevers, an enormous volume of clinical, epidemiological, serological, rickettsial and ecological data had accumulated by mid-1976. The original concept of using edge-punched cards for data recording and analysis was overwhelmed by the unexpected level of success. There are currently some 11,000 subjects/patients and nearly 24,000 serum samples. On each subject there is baseline demographic and social data and on patients there is additional clinical and therapeutic data. The sheer volume and the varying methods of data recording make current analysis impossible without ADP. Consideration was given, therefore, to conversion to ADP. After detailed assessment of the options available locally, a contract to purchase the hardware and initial software was signed on 14 May 1977. Systems analysis and the initial software preparation took place in the lag period to hardware delivery in late December 1977. As this was the first occasion that any medically orientated system had been installed within Malaysia, development was entirely from first principles and custom designed to unit requirements.

The specific requirements of the system involved not only the capacity for relatively massive data storage and manipulation but also a high level of flexibility to enable the completed basic system to cope with changing targets and needs in the research context.

The system is supplied by DATA GENERAL through a local agent and consists of a 64 K-byte central processing unit, dasher, 3 x visual display unit, 2 x 10 M-byte disc units (5 fixed and 5 removable for each), 1 x 9-track tape unit, and a 1 x 150 lines/min (nominal) line printer. An existing teletypewriter was modified and acts as a fourth in-put terminal. The system layout is shown diagrammatically in Figure 15.

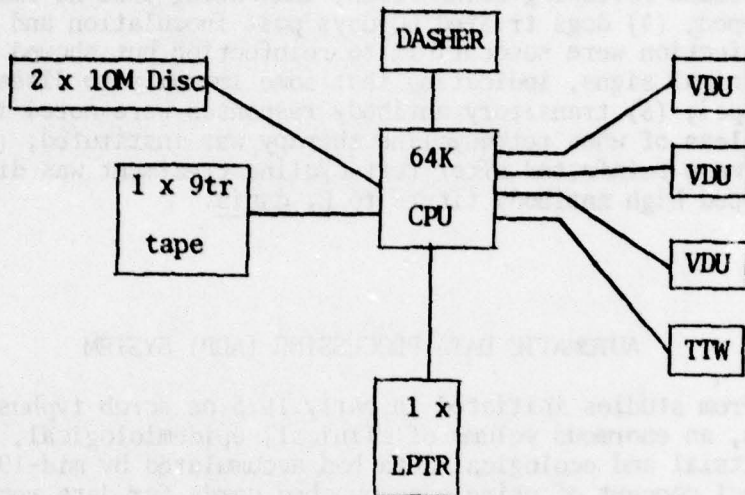


Figure 15. System configuration.

The dasher functions as system control, VDU's for interactive input/output, the TTW currently as a specifically dedicated input unit, and the 9-track tape unit for data dumping etc.

Data Base Description: Data base organization is in three types of data files. These are considered briefly below:

1. Main Primary Data Files: The main files are those considered to be basic to the type of research conducted by the unit, and are adaptable towards the attainment of a variety of targets. The main primary data files include:

A: Lab. animal file. Divided into two operational files

A1 - basic origin and admin data

A2 - recurrent clinical and experimental data

B: Human biochemistry file

C: Chigger direct fluorescent antibody test results

E: Environmental data

H: Human file: Divided into five operational files

H1 - basic identification and social data

H2 - clinical data opening an illness spell

H3 - laboratory data at opening of an illness spell

H4 - clinical/laboratory data during an illness spell

H5 - summary diagnostic and treatment data closing an illness spell

I: Rickettsial isolate source and characterization

M: Indirect fluorescent antibody test results (all sources)

N: Rodent/chigger data

P: Colony chigger pedigrees (not yet designed)

R: Rickettsial isolation data (mouse inoculation)

S: Infectious disease serology. Divided into two operational files

S1 - bacterial and protozoal serology

S2 - viral serology and specimen inventory

U: Chigger collection data

V: Collection site vegetation data

IR: Automatically derived summary of R and in turn generates basic entry for I if isolate positive.

ZB: Animal blood data. Divided into two operational files

ZB1 - haematology

ZB2 - biochemistry

2. Ancillary Primary Data Files: These are files designed for specific projects and, while being used in the same way as main data files currently, are less adaptable to alternative targets.

The ancillary primary data files include:

SUR: A general survey format for use with civilian or military population

ALS: A coordination file for antibody longevity or other long-term surveillance

PUO: A summary file similar in content to H2-H5 but less detailed. For use with old multiple format data or low intensity or uncontrolled studies. Divided into two operational files.

PUO 1 - initial clinical and laboratory data

2 - intermediate and summary data

BM: A summary file for the varied formats used in the Bukit Mendi survey. Suitable for future similar use

JS: Jengka longitudinal survey file. Divided into two operational files

INITIAL - base-line data at the first cross-sectional survey

REPEAT - data at all subsequent survey points

3. Secondary Data Files: These are temporary files that consist of re-organized, amalgamated, transformed and derived data from primary files. They are in fixed format and can be used as key files for further extraction from primary data or used directly for statistical comparisons and other appropriate analyses. These files are labelled as and when necessary.

Description of Software: It is not possible in this report to give other than an outline description of the software that executes the functions of the ADP system as a whole. It is designed to use two languages and to cover three broad functions considered below:

1. Housekeeping: These programs are in a variant of BASIC and cover the functions of file creation, deletion, amendment and listing by various options. (Note: in this context, each file sub-division is treated as a separate file).

2. Manipulation: These programs are, with occasional exceptions, in the same variant of BASIC and carry out the function of creating secondary files (using a large series of options) from one or more primary data files. These secondary files can then be listed or labelled and held. Certain file lists can be summarized.

3. Analysis: Some simple analysis programs (e.g. one-way tabulation) are in the same variant of BASIC, but most analyses and all of those involving mathematical manipulation are in FORTRAN IV. These analytical/statistical programs can be used on the fixed format secondary data files directly or, by using an option, can deal with direct input data. The unit is indebted to Mr. Richard See of NAMRU-2, Taiwan for making his MEDPAK and PORTSTATS packages freely available for adaptation to its needs.

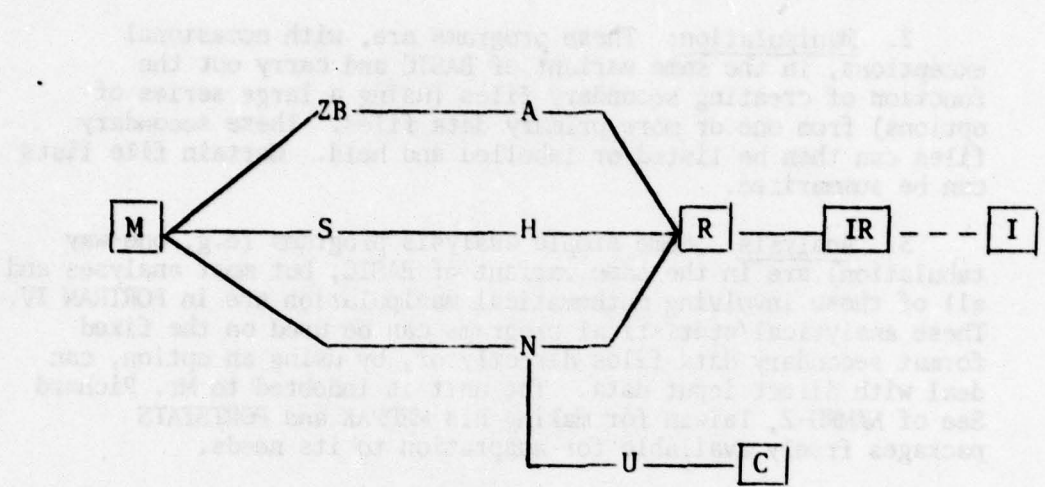
Utilization: In Figures 16-19, the utilization of the primary data files is shown, together with notes on additional linkages and the means by which this can be attained. For ease of comprehension, the charts are shown by unit department.

Current Status: This is considered from three aspects, the primary data files, the supporting software and the status of data input.

1. Primary Data Files: All the main and most of the ancillary primary data files are now on line. An idea of the magnitude of the task can be obtained from the size of the main files as of 31 August 1978 (Table 25).

2. Supporting Software: Of the nearly 400 programs required to support the envisaged system, some 300 are already written, entered and either functioning or under test. Remaining are those required for housekeeping of the smaller ancillary files and for the manipulation of ancillary files. The manipulatory programs for main primary data files are currently under test and will be proven by early October 1978. Despite the complexity of the interlocking system no unusual problems have been encountered in this area of development to date. It is anticipated that all software development of the envisaged system will be functional by 31 October 1978 or shortly thereafter. Minor modifications and developments are likely to continue for a further 2 months.

3. Data Input: The system uses as operators the unit personnel previously involved in manual record keeping. Training has been in-post and the same approach is being used as manipulative and analytical programs are tested and come on-line. Many, if not



☐ indicates department file

Figure 16. File utilization - Department of Rickettsiology

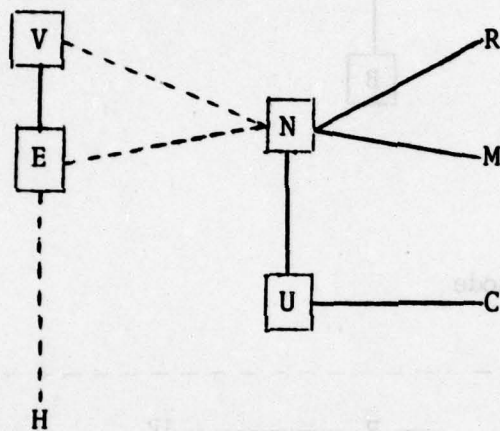
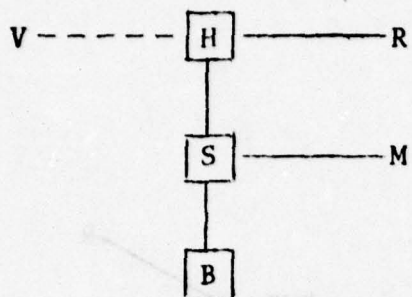
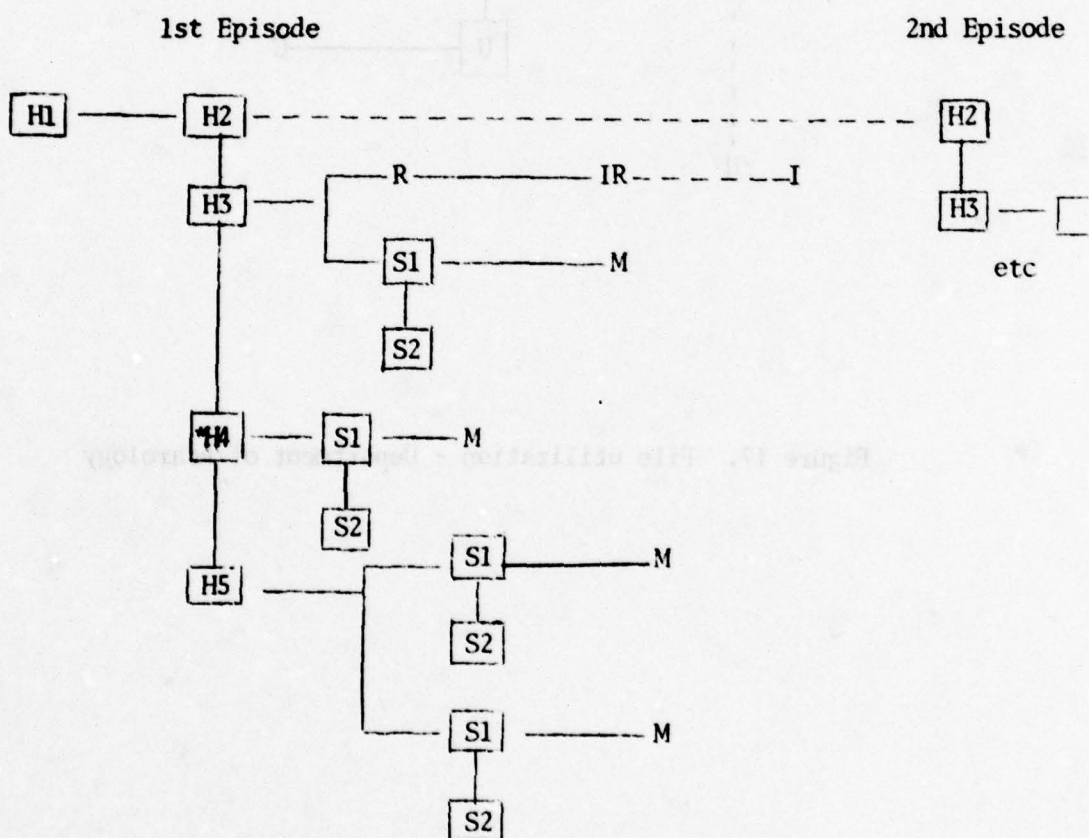


Figure 17. File utilization - Department of Acarology

a. System



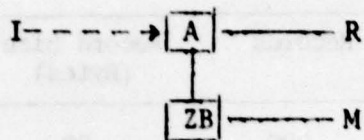
b. Flow Chart



*May be repeated as required before H5

Figure 18. File utilization - Department of Clinical Epidemiology

a. System



b. Flow Chart

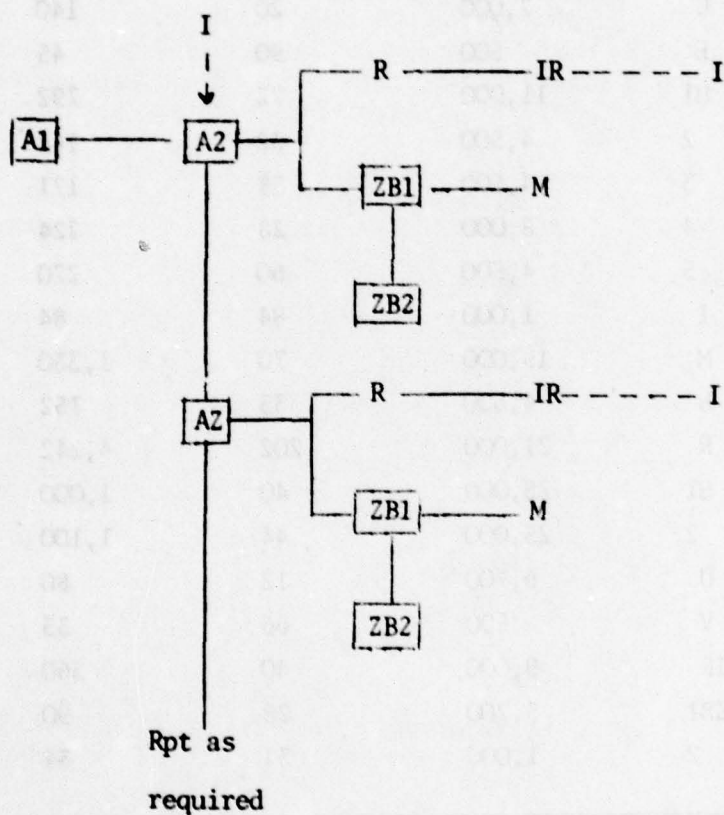


Figure 19. File utilization - Laboratory Animal Resources

Table 25. Status of main files as of 31 August 1978

File	Records	Record Size (Bytes)	Input (K-bytes)
A1	200	90	18
2/3	5,000	78	390
B	2,000	32	64
C	7,000	20	140
E	500	90	45
H1	11,000	72	792
2	4,500	32	144
3	4,500	38	171
4	8,000	28	224
5	4,500	60	270
I	1,000	84	84
M	19,000	70	1,330
N	4,600	33	152
R	21,000	202	4,242
S1	25,000	40	1,000
2	25,000	44	1,100
U	6,700	12	80
V	500	66	33
IR	9,000	40	360
ZB1	3,200	28	90
2	1,000	34	34
Total			10,763

most, of these operators have reached average professional speeds of $5-6 \times 10^3$ key-strokes per hour and their value is enhanced by their familiarity with the data and, thus, a below average error rate. This has meant that all departments are up to date with current data input and greater than expected inroads have been made into old data - the latter concerning only humans save for very minor exceptions.

Future Development: During the up-coming grant year the CPU is to be expanded to the maximum capacity of the current chassis - i.e. to 128 K-bytes or half the possible maximum overall. This will enable the system to operate simultaneously in two languages and better use the TTW as a second control module for FORTRAN. This modest expansion will enable the unit to maintain the momentum gained during the initial development - when deliberate policy gave priority to data input - into the definitive phase of analysis and beyond. The further advantage gained is the maintenance of the essential flexibility of the system.

Discussion: With the later than expected hardware delivery and some considered changes in approach (particularly the use of BASIC and FORTRAN) that delayed some implementation, the unit has achieved a great deal in the mere 8 months that progress has been possible. Indeed, a continued flexibility of approach has meant that in many if not most aspects, ADP development is in advance of the stage envisaged for this date.

We fully expect to be able to begin full and detailed analyses of old data and of several important current ones with the beginning of calendar 1979. The current system with its planned expansion should remain viable without other than minor hardware additions for several years.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 007 Field Studies of Rickettsioses and Other Tropical Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
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11. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AR A NUMBER	WORK UNIT NUMBER			
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12. TITLE (Precede with Security Classification Code) ^a							
(U) Tropical and Subtropical Diseases in Military Medicine							
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20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: US Army Medical Component, AFRIMS			
ADDRESS: Washington, DC 20012				ADDRESS: Bangkok, Thailand			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: RAPMUND, G., COL				NAME: SEGAL, H.E., LTC			
TELEPHONE: 202-576-3551				TELEPHONE: 281-7776			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
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				NAME: BURKE, D.S., MAJ			
				NAME: HARRISON, B.A., MAJ			
23. KEYWORDS (Precede EACH with Security Classification Code) (U) Infectious Diseases; (U) Dengue; (U) Hepatitis							
(U) Wound Infections; (U) Scrub Typhus; (U) Gonorrhea; (U) Vectors							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) To define the ecology and biology of tropical infectious diseases and to study the environmental variables that may affect the performance of US servicemen in tropical areas.</p> <p>24. (U) Routine diagnostic, epidemiological, serological, biochemical, microbiological, and entomological methods are being utilized. Field studies are emphasized and are supplemented by appropriate laboratory investigations.</p> <p>25. (U) 77 10 - 78 09 Dengue, hepatitis, influenza, war wound infections, scrub typhus, and gonorrhea were studied. Surveillance of dengue hemorrhagic fever (DHF) cases led to isolation of 37 Dengue-2, 11 D-3, and 19 D-4 strains. Virus in the blood of DHF patients was associated with the mononuclear cell fraction. A candidate D-2 vaccine was tested in Rhesus monkeys and found to be attenuated compared to wild virus. Longitudinal studies of hepatitis B infections disclosed an antigen prevalence of 9 percent in school children, and an 80 percent prevalence of hepatitis B surface antigen or antibody in the families of schoolage chronic carriers. Infection with Hepatitis B was transmitted to female gibbons with infectious human semen by parenteral and intravaginal routes. A mosquito bioassay for dengue viruses was developed employing Toxorhynchites splendens. An ecological and epidemiological study of dengue virus infections in a defined housing area was underway. Influenza surveillance led to the earliest isolations in Thailand of the H1N1 influenza A virus. Investigations in war wound infections and scrub typhus, describing the prevalence and incidence in Thai Army units, as well as associated ecological variables, were begun. Studies of the prevalence of B Lactamase-producing N. gonorrhea strains in Army and public health clinics were continued. Cytogenetic, electrophoretic, and morphological taxonomic studies were begun on Thai strains of Aedes aegypti and Anopheles balabacensis. For technical report see Walter Reed Army Institute of Research Annual Progress Report. 1 Oct 77-30 Sep 78.</p>							

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 008 Tropical and Subtropical Diseases in Military
Medicine (AFRIMS)

Investigators.

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1. Penicillin Resistant Neisseria gonorrhoeae Infection in Selected Clinic Populations

OBJECTIVES:

1. To study patients having a bacteriologically confirmed infection with N. gonorrhoeae.
2. To identify gonococcal infections resistant to penicillin and to demonstrate evidence of penicillinase production by chemical and culture techniques.
3. To attempt to relate laboratory findings to chemotherapeutic success or failure.
4. To refine and make locally available a simple and rapid screening technique for penicillinase production.
5. To investigate various methods of specimen collection and techniques for future study, to include the immunological aspects of this disease.

BACKGROUND: Between 1972 and 1974 an increasing resistance to penicillin of N. gonorrhoeae infections was demonstrated in Bangkok (1). During 1976, world-wide reports of isolations of penicillinase producing strains of N. gonorrhoeae suggested that a serious problem of great public health importance existed (2). The epidemiologic evidence shows an almost world-wide incidence of penicillinase producing strains and links many cases to travelers from and residents of Australia, Belgium, Canada, Denmark, Hong Kong, Japan, Korea, Singapore, Sweden, Switzerland, the United Kingdom, and the United States. West Africa and the Far East also appear to be significant factors in the spread of infection (2). Determination of penicillin resistance and penicillinase production are not routinely performed by most venereal disease clinics or physicians.

This Laboratory, in collaboration with the Royal Thai Army Hospital's venereal disease clinic, demonstrated that penicillinase production occurred in eight percent of 105 isolates over a seven month period and that 55 percent of the isolates had penicillin minimum inhibitory concentrations, MIC, of between 0.4 and 1.2 Units per milliliter. It was also demonstrated that 62% of patients reporting symptoms of gonorrhoeae were culture positive (3).

METHODS: Collaborative investigation efforts between our Laboratory, the Bangruk Hospital and the Royal Thai Army Hospital, have provided

a group of male and female patients for study. The Ban Chiwi Clinic, a subsidiary venereal disease unit of the Bangruk Hospital, is the sources of female patients and the Royal Thai Army Hospital's Venereal Disease Clinic is the source of male patients. Patients were selected as demonstrating clinical symptoms of Neisseria gonorrhoeae infection. Patients whose infections were resistant to treatment were followed (4).

N. gonorrhoeae (5) strains isolated from patients were collated with MIC testing data and with the production of penicillinase as demonstrated by the penicillin plate inhibition method (6), and by the iodometric and cephalosporin techniques (7). Experience with the iodometric and cephalosporin methods for penicillinase production as well as with the culture plate sensitivity technique allowed laboratory personnel to refine and make locally available the culture plate technique for rapid screening.

In addition to specimens for bacteriological identification, serum and vaginal washings were obtained for future investigation of the immunological aspects of the disease. Attempts will be made to correlate with case history files, penicillinase production, and penicillin MIC trends. Lyophilized isolates from all cases were retained.

Table 1

Male (n=8)		Female (n=9)	
Case	MIC(U/ml)	Case	MIC(U/ml)
A2	40	BC30	10
A5/A19	40	BC43	40
A47	40	BC53	40
A49	10	BC94	10
A61	40	BC189	40
A78/A83	40	BC192	40
A131	40	BC194	40
A132	40	BC209	5
		BC211	40

Table 2. Selected Case Histories

- Case A2: 21 yrs., male, single, symptomatic 3 days after contact exposure. Culture positive N. gonorrhoea
- 1st Treatment: Pen. G sodium 5 MU(IM), Probenecid 1 gm. (oral)
3 days after, pus occurred, no contact exposure
Culture positive N. gonorrhoea
- 2nd treatment: Kanamycin 2 gm. (IM) 1 dose
1 day after, asymptomatic
Culture negative N. gonorrhoea
- Case A5/A19: 32 yrs., male, chronic urethritis for 2-3 weeks, last contact exposure over one month
Culture positive N. gonorrhoea
- 1st Treatment: Pen. G sodium 5 MU(IM), Probenecid 1 gm. (oral)
1 week later, discharge and dysurea
Culture positive N. gonorrhoea
- 2nd Treatment: Kanamycin 2 gm(IM) 1 dose
1 month later, asymptomatic
Culture negative N. gonorrhoea
- Case BC192: 20 yrs., bar girl, leukorrhea and itching, 2-3 days
Smear positive N. gonorrhoea, no culture taken
- 1st Treatment: Probenecid 1 gm. (oral), Ampicillin 3.5 gm. (oral)
1 day later, leukorrhea
Smear and culture positive N. gonorrhoea
- 2nd Treatment: Probenecid 1 gm. (oral), Ampicillin 3.5 gm. (oral)
1 day later, no signs, symptoms present
Smear negative N. gonorrhoea, no culture taken
- 3rd Treatment: Probenecid 1 gm. (oral), Ampicillin 3.5 gm. (oral)
20 days later, lower abdominal bilateral pain, 2 days
Smear negative N. gonorrhoea, no culture taken
- 4th Treatment: Trobicin (Sepectinomycin dihydrochloride 2 gm. (IM)
1 day later, symptoms diminishing
Smear negative N. gonorrhoea
- 5th Treatment: Trobicin 2 gm. (IM)
No patient follow-up

Case BC 209: 21 yrs., bar girl, leukorrhea, 1 day
Smear and culture positive N. gonorrhoea

1st Treatment: Probenecid 1 gm. (oral), Ampicillin 3.5 gm. (oral)
 1 day later, leukorrhea
Smear negative N. gonorrhoea

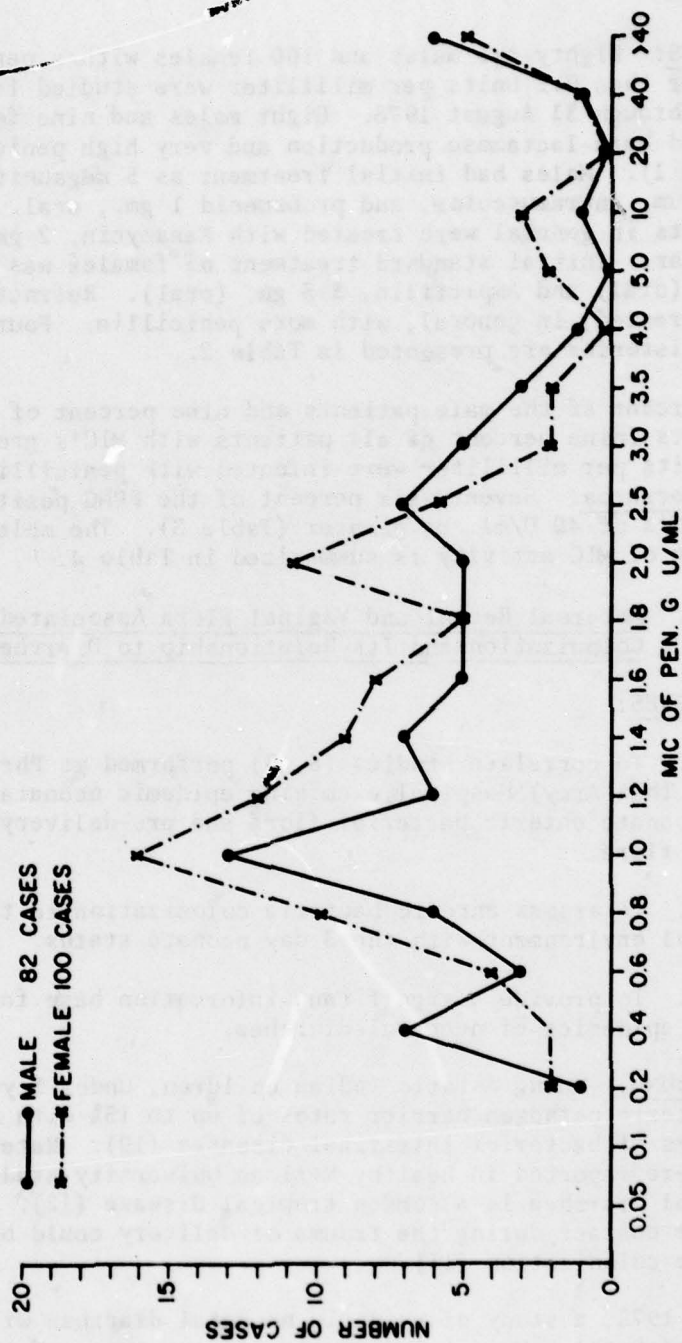
2nd Treatment: Probenecid 1 gm. (oral), Ampicillin 3.5 gm. (oral)
 18 days later, leukorrhea
Smear negative N. gonorrhoea
Culture positive N. gonorrhoea

3rd Treatment: Trobicin 2 gm. (IM)
 1 day later, leukorrhea
Smear and culture negative N. gonorrhoea

Table 3. Pattern of Pen. G MIC (U/ML) Activity

MIC Pen. G U/ml.	MALE (n=82) Cases	FEMALE (n=100) Cases
0.05	0	0
0.1	0	0
0.2	1	2
0.4	7	2
0.6	3	4
0.8	6	10
1.0	13	16
1.2	6	12
1.4	7	9
1.6	5	8
1.8	5	5
2.0	5	11
2.5	7	6
3.0	5	2
3.5	3	2
4.0	1	0
5.0	0	2
10.0	1	3
20.0	0	0
40.0	1	1
40.0	6	5

Table 4. MINIMUM INHIBITORY CONCENTRATION (MIC) PATTERN



RESULTS: Eighty-two males and 100 females with a penicillin MIC greater than 0.2 Units per milliliter were studied from 13 March 1978 through 31 August 1978. Eight males and nine females demonstrated beta-lactamase production and very high penicillin MICs (Table 1). Males had initial treatment as 5 megaunits of penicillin G sodium, intramuscular, and probenecid 1 gm., oral. Refractory patients in general were treated with Kanamycin, 2 gm., intramuscular. Initial standard treatment of females was Probenecid, 1 gm. (oral) and Ampicillin, 3.5 gm. (oral). Refractory patients were treated, in general, with more penicillin. Four representative case histories are presented in Table 2.

Ten percent of the male patients and nine percent of the female patients (nine percent of all patients with MIC's greater than 0.2 Units per milliliter were infected with penicillinase producing N. gonorrhoea. Seventy-six percent of the PPNG positive patients had MIC's of 40 U/ml. or greater (Table 3). The male and female pattern of MIC activity is summarized in Table 4.

2. Maternal Rectal and Vaginal Flora Associated with Infant Colonization and Its Relationship to Diarrhea

OBJECTIVES:

1. To correlate studies (8, 9) performed at Phra Mongkutklao (Royal Thai Army) Hospital examining epidemic neonatal diarrhea with neonate enteric bacterial flora and pre-delivery maternal rectal flora.
2. To assess enteric bacteria colonization in the pre-delivery maternal environment with the 3 day neonate status.
3. To provide a significant information base for investigating future epidemics of neonatal diarrhea.

BACKGROUND: Young Asiatic Indian children, under 5 years of age, had enteric pathogen carrier rates of up to 13% with no signs or symptoms of bacterial intestinal diseases (10). Rates of 27 percent were reported in healthy Mexican University students (11). Neonatal diarrhea is a common tropical disease (12). The maternal-neonate contact during the trauma of delivery could be a source of neonate colonization (13).

During 1975, a study of epidemic neonatal diarrhea with sepsis at Phra Mongkutklao Hospital demonstrated serotypes of enteropathogenic

E. coli (EPC) in symptomatic infants in 20 percent of the nursing staff. Subsequent nursing and patient management changes led to a marked decrease in cases (9). A random follow-up study in 1977 on 103 mothers upon admission to the delivery room found a 48 percent rectal colonization of medically significant bacteria of which 64 percent were serotypes of generally recognized enteropathogenic Escherichia coli (8).

METHODS: The study was performed from 21 February to 25 April 1978 at the nursery and delivery room of Phra Mongkutklao Hospital. Three hundred ninety-one maternal-neonate pairs were studied. A pair is defined as one mother and her newborn; a case is one individual of a pair. Rectal and vaginal cultures were performed on mothers upon admission to the delivery room. History was acquired at time of specimen collection. Standard enteric bacterial isolation, identification, and serotyping was performed (14,15,16). Rectal culture of infants were performed at 24 and 72 hours after birth and when associated with diarrhea. Follow-up infant diarrhea history after the 3rd day of discharge was attempted through the hospital post-natal clinic. Infant specimens were not acquired after 72 hours post delivery.

RESULTS: Twenty-six (6.65%) of 391 maternal-neonate pairs had identical enteric isolates. Ninety-six percent of the paired isolates were E. coli of serotypes 018a 018c:K 77, 055:K 59, 0111:K 58, and 086a (Table 5). Maternal ages of these 26 cases were 17-33 years and parities 1-6. Twenty of the mothers resided outside of Bangkok (Table 6).

Infants and mothers were followed after 72 hours at the post-natal clinic. Treatment for diarrhea was administered as appropriate to symptoms without cultures being taken. Fifteen infants had diarrhea after 72 hours but prior to discharge. Mothers of this study, upon discharge, were also given survey questionnaires to respond to should their infants become ill during the first week after discharge. Twenty-five mothers indicated that the infants did develop diarrhea. Sixty-one (16%) of the 391 mothers also had a recent (within 3 days), prior history of diarrhea, and 37 of the 62 (60%) were found positive for enteric isolates (Table 7).

Sixty-nine percent of the maternal only isolates (Table 8) were of E. coli serotypes 018a 018c:K 77, 0111:K 58, and 055:K 59. Ninety-two percent of the matched pair isolates were also of these serotypes (Table 5). Seventy-three percent of infant isolates which were not associated with maternal isolates were of these serotypes (Table 9).

Table 5. Maternal-Neonate Enteric Bacteria Matched Isolates of 391 Paired Cases

	Maternal Source			Total PAIRED Isolates	%PAIRED Isolates	%PAIRED Cases
	Rectal	Vaginal	R+V			
<u>E. coli</u>						
018a 018c:K77	5	5	2	12	46	3.07
0111:K58	5	1		6	23	1.53
055:K59	3	2	1	6	23	1.53
086a			1	1	4	0.26
Enteropathogenic <u>E. coli</u> Total				25	96	6.39
Salmonella group El supp.			1	1	4	0.26
Matched isolate total:				26		6.65

Table 6. Maternal History of Matched Neonate-Maternal Isolates (26 Cases)

Matched Isolates	#Cases	Age/#Cases	Parity/	Home Province	
			#Cases	Bangkok	Other
<u>E. coli</u>					
018a 018c:K77	12	18/1, 19/1, 20/1 21/2, 22/2, 25/2 24/1, 27/2	1/3,2/6 3/2,1-unk	1	11
055:K59	6	17/1, 18/1, 20/1 28/1, 33/1	1/3,3/2	2	4
0111:K58	6	18/1, 20/1, 25/1 27/2	1/2,2/4	2	4
086a	1	29/1	1/1	1	0
<u>E. coli</u> total:	25				
Salmonella group El spp.	1	24/1	3/1	0	1
Isolate total:	26				

Table 7. Sixty-one Symptomatic Maternal Diarrhea Cases of 391 Mother-Neonate Paired Cases (No symptomatic infants)

Group I	Maternal-Neonate isolate match.	
<u>E. coli</u>	018a 018c:K 77	2 cases
	055:K 59	1 case
Group II	Maternal isolate only, infant negative.	
<u>E. coli</u>	055:K 59	3 cases
	0111:K 58	2 cases
	018a 018c:K 77	1 case
	0127a	1 case
Group III	Infant isolate only, mother negative.	
<u>E. coli</u>	028:K 73	1 case
	0111:K 58	2 cases
	0111:K 58, 086a	1 case
	020a 020b:K 84	1 case
	018a 018c:K 77	10 cases
	018a 018c:K 77, Shig. boydii 15	1 case
	0125:K 70	1 case
	055:K 59	5 cases
	086a	2 cases
	018a, Shig. A-D 01	1 case
Salmonella gr. El spp.		2 cases
		37 cases
Group IV	Mother and infant negative for bacterial isolates	
	Symptomatic only	24 total cases

Table 8. E. coli Maternal Isolates not Matched with Neonates
(391 total cases)

	Isolate Source		R+V	Total Isolates	% <u>E. coli</u> Isolates	% Cases
	Rectal	Vaginal				
<u>E. coli</u>						
018a 018c:K 77	15	9		24	32.00	6.14
0111:K 58	13	3	1	17	22.67	4.35
055:K 59	6	3	2	11	14.67	2.81
0127a	5			5	6.67	1.28
0125:K 70	1	3		4	5.33	1.02
086a	1	2		3	4.00	0.76
020a 020b:K 84	1	2		3	4.00	0.76
044:K 77		1		1	1.33	0.26
0128:K 67		2		2	2.67	0.51
025	1			1	1.33	0.26
026:K 60	1			1	1.33	0.26
0127a:K 63			1	1	1.33	0.26
020a 020c:K 61	1			1	1.33	0.26
044:K 74	1			1	1.33	0.26
Total <u>E. coli</u>				75		19.18
Total <u>E. coli</u> cases				72		18.41

Cases with two species EPEC

<u>E. coli</u> 0127a	Rectal	1 case
044:K 77	Vaginal	
055:K 59	Rectal	1 case
0111:K 58	Vaginal	
0111:K 58	Rectal	1 case
018a 018c:K 77	Vaginal	

Table 9. Neonate *E. coli* Isolates Not Matched with Mothers of 391 Paired Cases (All infants diarrhea asymptomatic)

	Total Isolates	% <i>E. coli</i> Isolates	% Cases
018a 018c:K 77	28	38.36	7.16
055:K 59	13	17.81	3.32
0111:K 58	12	16.44	3.07
086a	4	5.48	1.02
0125:K 70	3	4.11	0.77
020a 020b:K 84	3	4.11	0.77
0127a	2	2.74	0.51
0126:K 71	2	2.74	0.51
0119:K 69	1	1.37	0.26
018a 018c:K 67	1	1.37	0.26
028:K 73	1	1.37	0.26
044:K 74	1	1.37	0.26
026:K 60	1	1.37	0.26
0128:K 67	1	1.37	0.26

(3 cases had two species of EPEC)

Table 10. Maternal-Neonate Enteric Bacterial Matched Isolates of 391 Paired Cases

	24 hrs. Isolates	72 hrs. Isolates	24+72 hrs. Isolates	Total Paired Isolates
<u><i>E. coli</i></u>				
018a 018c:k 77	6	4	2	12
0111:K 58	4	2		6
055:K 59	4	2		6
086a	1			1
<u><i>E. coli</i> totals</u>				
Salmonella group El spp.		1		
Total	15	9	2	26

Table 11. Isolates of 12 Mother-Infant Pairs, Unmatch,
of 391 Mother-Infant Paired Cases

<u>Mother</u>	<u>Infant</u>
<u>E. coli</u> 0112a 0112c:K 66	Salm. gr. E1 spp.
020a 020c:K 61	Salm. gr. E4 spp.
086a	Salm. gr. E1 spp.
018a 018c:K 77	Salm. gr. E1 spp.
0111:K 58	Salm. gr. E4 spp.
0127a, 0111:K 58	Sh. boydii 15 and <u>E. coli</u> 018a 018c:K77
0127a:K 63	<u>E. coli</u> 0111:K 53 and <u>E. coli</u> 018a 018c:K 77
0111:K 58	Salm. gr. E1 spp. + <u>E. coli</u> 055:K59 and <u>E. coli</u> 020a 020b:K 84
<u>E. coli</u> 018a 018c:K 77, Salm. gr. E1 spp	<u>E. coli</u> 0111:K 58
Sh. boydii 2	Salm. gr. E4 spp. + <u>E. coli</u> 018a 018c:K 77
V. parahaemolyticus	Salm. gr. E spp. + <u>E. coli</u> 018a 018c:K 77
V. parahaemolyticus	<u>E. coli</u> 055:K 59

Table 12. Maternal Enteric Bacterial Isolates (391 Pairs)

	# Cases	Age/#Cases	Parity/ #Case	Home Province Bangkok	Out
<i>E. coli</i> 018a 018c:K 77	24	16/1,20/4,21/2,22/2,23/2 25/3,26/2,27/2,28/1,29/1 30/1,32/2,35/1	1/15,2/4 3/5	5	19
0111:K 58	17	18/2,19/2,20/1,21/3,22/1 23/1,24/1,25/2,26/1,28/1 30/1,35/1	1/9,2/6 4/2	4 1-unk	12
055:K 59	11	17/1,18/2,23/2,26/1,28/2 30/1,31/1,32/1	1/6,2/3 3/1,4/1	4	7
0127a	5	21/1,24/1,27/2,35/1	1/3,2/1 4/1	2	3
0125:K 70	4	20/1,23/1,25/1,28/1	1/1,2/1 3/1	2	2
020a 020b:K 84	3	23/1,30/1,32/1	2/1,3/1 1-unk	0	3
086a	3	21/1,22/1,27/1	1/1,2/1 3/1	1	2
044:K 74	2	21/1,37/1	2/1,4/1	0	2
0128:K 67	2	20/1,29/1	1/2	0	2
0127a:K 63	1	23/1	2/1	0	1
026:K 60	1	19/1	1/1	0	1
020a 020b:K 61	1	16/1	1/1	0	1
025	1	26/1	unk	0	1
Salm. gr. El spp.	2	22/1,23/1	2/2	1	1
<i>S. paratyphi</i> A	1	20/1	2/1	0	1
<i>S. somniiform</i> I	1	21/1	1/1	0	1
<i>Shigella</i> A-D 02	1	22/1	1/1	0	1
<i>E. coli</i> total:	75				
Isolate total:	80				

The E. coli serotypes 018a 018c:K 77, 9111:K 58, and 055:K 59 were the predominate organisms isolated from mothers infants and mother-infant matched cases. Twenty-six of 61 (42%) symptomatic of which 37 (70%) were positive for E. coli of these 3 serotypes (Table 7).

Isolates from infants at 24 hours, 72 hours and found at both 24 and 72 hours are summarized in Table 10. Of E. coli serotypes, 018a 018c:K 77, 0111:K 58, 955:K 59 and 086a; 50%, 50%, 67%, 67% and 100% respectively were isolated at 24 hours. Twelve mother-infant pairs had significant isolates which were not the same organisms (Table 11).

Maternal isolates and age; parity, and home province history are summarized in Table 12.

3. Ectoparasite and Rickettsia tsutsugamushi Studies in Thailand

OBJECTIVE: To establish and describe the chiggers and ticks that are vector; or potential vectors of human pathogens in Thailand, and to determine the geographical distribution of Rickettsia tsutsugamushi in natural populations of chiggers in Thailand.

BACKGROUND: This is a continuing project that began in the early 1960's. Emphasis during the early years was placed on establishing rodent-ectoparasite associations and collecting, classifying and determining the distribution of chiggers and ticks in Thailand. This early work served as the basis for a number of publications, of which Lakshana (17) and Lekagul and McNeely (18) have established a very firm taxonomic base on which epidemiological studies on Rickettsia tsutsugamushi in Thailand can proceed. More recent emphasis has now shifted to the distribution and strains of R. tsutsugamushi that occur in vector chiggers in Thailand. However problems still exist in identifying chigger specimens collected in Thailand, hence taxonomic studies on chiggers are continuing.

METHODS: Ectoparasites are collected from live trapped rodents and other small mammals by removal with forceps, by scraping or by holding the animals alive over a pan of water and allowing engorged ectoparasites to drop into the water. Engorged chiggers are normally preserved in alcohol and mounted on slides for study. Chiggers used for R. tsutsugamushi isolation attempts are preferably unengorged. Unengorged chiggers are usually found in leaf litter, on rotten logs and other favorable habitats frequented by rodents and other small mammals, and are easily collected by using 5" x 5"

formica black plates. Collected unengorged chiggers are placed and kept alive in vials of water, which are then shipped to USAMRU-Kuala Lumpur for rickettsia isolation. A technique using direct immuno-fluorescence has recently been developed to detect rickettsia in naturally infected mites (19). Using this technique, the internal contents of each unengorged chigger can be screened for nine different strains of R. tsutsugamushi, i.e., Karp, Gilliam, Kato TC 586, TA 678, TA 686, TA 716, TA 763 and TH 1817. After the internal contents of the chigger has been tested for rickettsia, its exoskeleton is mounted in Holyer's mounting media on a slide for identification.

RESULTS: Between June 1977-August 1978, 2,251 unengorged chiggers collected in Thailand by the black plate method, were identified and sent to USAMRU-Kuala Lumpur for rickettsia isolation (Table 13). These specimens were collected in the following provinces: Chiangmai, Kanchanaburi, Nakhon Ratchasima, Prachinburi, Surin and Ubon Ratchathani. A total of 13 species of chiggers in 5 genera were involved, with Leptotrombidium (L.) deliense, the primary R. tsutsugamushi vector in Thailand being the most abundant species. The results of the rickettsia isolation attempts are shown in Table 2. Slightly over 10% of the chiggers screened for R. tsutsugamushi were positive. The chigger species found positive for R. tsutsugamushi are listed in Table 15. Leptotrombidium (L.) deliense, the most commonly collected species, also yielded the most rickettsia isolations. The other species positive for rickettsia were: Leptotrombidium (L.) miculum arvinum, Microtrombicula chamlongi and two new undescribed species collected in Chiangmai, Leptotrombidium (L.) species A and species B. Data on R. tsutsugamushi strains found in Thailand will be described in the Annual Report from USAMRU-Kuala Lumpur.

In December 1977 a total of 343 Leptotrombidium (L.) deliense, 251 L. (L.) scutellare and 85 L. (L.) striatum were collected engorged from field rodents and shipped alive to the Walter Reed Army Institute of Research. These specimens were for ongoing research requiring progeny broods.

Reports of twelve new species of Leptotrombidium collected in Thailand are currently being prepared for publication. One of the new species is very similar to L. (L.) deliense, the primary vector of R. tsutsugamushi in Thailand. An additional 4 new species of Leptotrombidium and one new species of Gahrliepia were collected in the above black plate collections. A checklist of the ticks of Thailand is currently in manuscript form.

Table 13. Unengorged Chiggers Collected in Thailand between June 77 - September 78, and sent to USAMRU - Kuala Lumpur for Rickettsia Isolations.

Chigger Species	LOCATIONS						TOTAL
	Chiang Mai	Kanchanaburi	Nakhon Ratchasima	Prachin Buri	Surin	Ubon Ratchathani	
No. of Collections	8	9	12	3	4	9	45
<u>Gahrliepia</u> (G.) species A*	-	-	-	-	2	-	2
<u>Lept.</u> (L.) <u>deliense</u>	13	90	543	1	145	949	1,741
" " <u>fulleri</u>	-	-	21	-	-	-	21
" " <u>miculum arvinum</u>	46	4	8	-	-	-	58
" " species A	119	-	-	-	-	-	119
" " species B	4	-	-	-	-	-	4
" " species C	-	-	-	-	15	-	15
" " species D	1	1	-	-	-	-	2
<u>L. (Trom.) paniculatum</u>	-	1	-	9	-	-	10
<u>Microtrombicula</u> <u>chamlongi</u>	-	-	5	-	-	1	6
<u>Siseca rara</u>	-	-	16	-	-	-	16
<u>Walchiella oudemansi</u>	-	-	68	-	-	-	68
" <u>traubi</u>	-	-	1	-	-	-	1
TOTAL	183	96	662	10	162	950	2,063

* Species with alphabetical designations are new and undescribed.

Table 14. *Rickettsia tsutsugamushi* Isolations from Thai Chiggers.*

Location	Total Chiggers Examined	Number Infected	Percent Infected
Chiang Mai	133	26	19.55
Nakhon Ratchasima	49	31	63.27
Prachin Buri	1	1	100.0
Ubon Ratchathani	579	26	4.5
Total	762	84	11.02

* The collections from Kanchanaburi, Surin and several collections from Khao Yai National Park have been deleted due to technical problems.

Table 15. Source and species of Thai Chiggers Infected with Rickettsia tsutsugamushi.

Location	Chigger Species	Number Screened	Chiggers Infected	
			Number	Percent
Chiang Mai	<u>L. (L.) deliense</u>	52	12	23.08
	" " <u>miculum arvinum</u>	37	5	13.51
	" " species A	39	7	17.95
	" " species B	4	2	50.00
	" " species D	1	0	0
Nakhon Ratchasima	<u>L. (L.) deliense</u>	47	30	63.83
	<u>Microtrombicula chamlongi</u>	2	1	50.00
Prachin Buri	<u>L. (L.) deliense</u>	1	1	100.00
Ubon Ratchathani	<u>L. (L.) deliense</u>	579	26	4.49
	Totals	762	84	11.02

4. Mosquito Cytogenetic and Electrophoresis Studies

OBJECTIVE: To define and delimit the species or strains of mosquito species in Thailand that serve as the primary vectors of human pathogens by cytogenetic and enzymatic techniques for:

1. a check against current morphological species concepts;
2. the accurate determination of the limits of gene pools in natural populations of vector species; and
3. the correlation of genetic variation in natural populations of the primary vectors with the degree of susceptibility to dengue viruses and human malaria.

BACKGROUND: Considerable evidence now exists that different biological species of organisms, as defined by Mayr (20), may exist in the absence or near absence of definable morphological differences in all or most life stages. Such cryptic or sibling species may be distinct only on the basis of ecological, behavioral and/or cytogenetic differences. The occurrence of such species in important vector species groups of mosquitoes is well documented (21, 22), and their discovery is essential for developing effective control programs and understanding the epidemiology of vector-borne diseases (23). In addition, the ability of mosquito species or strains of species to transmit certain human pathogens has been shown to be under genetic control (24, 25, 26, 27). Cytogenetic and electrophoresis techniques have been shown to be effective for determining the genetic variability (including disease susceptibility) in natural populations of mosquitoes (28). These techniques, coupled with morphological studies and hybridization experiments, provide the most well founded basis for species and vector strain differentiation.

METHODS: Initially, colony strains (Table 16) of Aedes aegypti, albopictus, malayensis, Anopheles balabacensis and maculatus were utilized to develop facilities, train personnel and to standardize the cytogenetic and electrophoresis techniques. The cytogenetic techniques employed were: (1) larval salivary polytene chromosome preparations by a modification of the standard chromosome squash technique (29); (2) larval brain metaphase preparations by a modified technique based on Baimai (30); and (3) ovarian nurse cell polytene chromosome preparations from adult females by the method described by Coluzzi (31). The electrophoresis techniques employed were those of Steiner and Joslyn (32).

Chromosome maps and electrophoresis starch-gel esterase patterns will be initiated for selected laboratory colony strains or species. These maps or patterns will serve as "standards" for later comparisons with other strains and/or species. Once "standard" maps or esterase patterns have been established, wild collected populations will be sampled to survey the variations occurring in natural populations. Wild and/or colony strains of currently recognized morphological species that exhibit sufficient cytogenetic and/or electrophoretic differences will be studied further by hybridization experiments to determine if they are conspecific. Strains of aegypti (wild or colony) that exhibit distinct esterase polymorphisms will be tested for susceptibility to infection with dengue viruses.

RESULTS: Squash preparations of the salivary glands of 4th stage larvae produced mixed results. Anopheles balabacensis larvae yielded fairly good results with 2-4% of the slides having well-spread chromosome arms. Chromosome preparations of An. maculatus were less favorable and those for Aedes aegypti were a complete failure. The chromosomes of balabacensis have been photographed and work has begun on the development of a standard chromosome map for this species.

Preparations of metaphase chromosomes from larval brains have been quite successful. A study to compare the different karyotypes is in progress.

The development of electrophoresis techniques was expedited by Dr. W.W.M. Steiner, University of Illinois, who spent one month at Mahidol University training personnel and refining his techniques for Ae. aegypti and anophelines. Field collected specimens of the Southeast Asian Anopheles hyrcanus complex were selected to evaluate the ability of electrophoresis techniques to differentiate very closely related species. According to morphological characteristics, this group is a very closely related assemblage of sibling species, of which at least 8 species occur in Thailand (33). A total of 66 nigerrimus, 148 peditaeniatus and 140 sinensis females were collected from 4 widely separated localities within a 100 km. radius of Bangkok, and analyzed for 15, 16 and 16 enzymes respectively. Based on esterase activity, these species exhibited very similar patterns of gene variation, with some polymorphism at different loci. Species patterns were distinct for some loci, which substantiates the current concept that these are very closely related members of a sibling species complex. Further analysis of these data is in progress and additional specimens of these and other species in the complex are being collected for further investigations.

Table 16. Colony strains of Aedes and Anopheles mosquitoes used in cytogenetic and electrophoresis studies.

Colony - species	Strain #	Date of Collection	Age of Colony	Location of original collection	Numbers to start strain
<u>Aedes aegypti</u>	1	June - July 1968	122 months	Koh Samui (Island) Surat Thani, Thailand	?
"	3	Aug. - Sept. 1977	12 months	Bangkok, Thailand	many larvae
"	4	November 1977	9 months	Bangkok, Thailand	1 ♀
"	5	June 1978	2 months	Chiang Mai, Thailand	40 [±] larvae
<u>Aedes albopictus</u>	2	May 1978	3 months	Chon Buri, Thailand	many larvae
<u>Aedes malayensis</u>	1	20 April 1968	124 months	Prachuap Kiri Khan, Thailand	many larvae
<u>Anopheles balabacensis</u>	1	February 1964	174 months	Khao Mai Khaeo, Chon Buri, Thailand	many adults and larvae
<u>Anopheles maculatus</u>	1	1966	144 [±] months	Colony - Kuala Lumpur, Malaysia	?

Table 17. Relative frequencies of esterase alleles at the Est-1 and Est-2 Loci in larvae of 3 colonies of Aedes aegypti and colonies of Aedes albopictus and Ae. malayensis.

Locus	Mosquito Colonies - Strains	Allele					
		.92	.94	.96	.98	1.00	1.02
Est - 1	aegypti - 1	-	-	-	-	.70	.30
	aegypti - 3	-	-	-	-	.89	.11
	aegypti - 4	-	-	-	.53	.43	.03
	albopictus-2	-	-	.02	.39	.59	-
	malayensis-1	.30	.40	.30	-	-	-
Est - 2	aegypti - 1	-	-	-	1.00	-	-
	aegypti - 3	-	-	-	-	1.00	-
	aegypti - 4 ¹	-	-	-	-	Null?	-
	albopictus-2 ²						
	malayensis-1 ²						

¹ This colony has the Est-1, .98 allele overlapping the Est-2 zone of activity, making it difficult to assess the presence of no activity.

² Enzyme activity too low to accurately diagnose the banding patterns.

A total of 22 enzymes were tested against 50 larvae each of four colony strains of Aedes aegypti and a total of six esterases were detected, although most colonies exhibited only four esterases. The relative frequencies of esterase alleles at the Est-1 and Est-2 loci in 50 larvae each of three strains of aegypti and 2 closely related Stegomyia species, albopictus and malayensis, are tabulated in Table 17. These data demonstrate the ability of electrophoresis techniques to detect esterase and esterase allele differences between closely related strains (Thai) of aegypti and closely related species of Aedes (Stegomyia) mosquitoes. The data for the other four esterases detected in these colony strains are being analyzed. Additional strains of aegypti will be tested in the future in preparation for the selection of strains to test for susceptibility to dengue viruses.

All aspects of this project will be continued, except for the attempted slide preparation of polytene chromosomes from the larval salivary glands of Ae. aegypti.

5. Mosquito Survey and Taxonomic Studies

OBJECTIVE: To elucidate the mosquito fauna of Thailand and Southeast Asia, with primary emphasis on the identification of diagnostic characters for the separation of vector species and groups containing vector species of human pathogens.

BACKGROUND: This is a continuation of collaborative efforts begun in the 1960's with the Southeast Asia Mosquito Project (SEAMP), Smithsonian Institution, and currently continued with the Medical Entomology Project (MEP), Smithsonian Institution, Washington, D.C. Earlier efforts were primarily concerned with general surveys for elucidating the mosquito fauna of Thailand. However, by 1967-68 general surveys were discontinued and studies were aimed at target species or species groups of suspected or known vectors of human pathogens. During the period 1970 to the present a large number of excellent taxonomic studies on the mosquitoes of Thailand and Southeast Asia have been published under the auspices of SEAMP, MEP and the U.S. Army Medical Component, SEATO. Despite this progress, serious identification problems, involving suspected and known mosquito vectors of arboviruses, filariasis, and malaria still exist in Thailand. These problems, in conjunction with the resurgence of malaria in Thailand, justify the continuation of basic taxonomic studies.

METHODS: Surveys for target species and species groups are conducted at selected sites and habitats throughout Thailand. These surveys

involve the collection of immature and adult mosquitoes, with emphasis placed on reared adults with associated larval and pupal skins, and on progeny adults (with associated larval and pupal skins) reared from eggs oviposited by known wild collected females. Specimens are curated and pinned or slide mounted for further study by investigators in the laboratory, or shipped for study to MEP or other world recognized authorities. Studies primarily consist of an analysis of intra-interspecific variations to identify useful characters for separating the species. Useful diagnostic characters, new species records and new taxa found in Thailand are prepared for publication and described in scientific journals.

RESULTS: Numerous progeny broods of species in the Aedes (Finlaya) niveus group, collected from Kanchanaburi Province, were curated and sent for study to Dr. Kenneth L. Knight, North Carolina State University. At least two new species were involved and one species was recently described by Knight (34). This new species, harinasutai, is the species incriminated (as Aedes niveus group) as the primary vector of subperiodic Wuchereria bancrofti parasites in man in Kanchanaburi Province by Harinasuta et al. (35).

A large collection of larvae and reared adults with associated immature skins, of a new species and probable new subgenus of Aedes from Kanchanaburi Province, were curated and sent for further study to the Medical Entomology Project (MEP), Smithsonian Institution. In addition, over 4,600 slide preparations of mosquito immatures from Columbia and Panama were mounted and labeled at the request of MEP, and returned to that project for further study.

More than 20 progeny broods of Anopheles balabacensis have been reared with associated immature skins. These specimens are being curated and prepared for shipment to E.L. Peyton (MEP), who is currently revising the leucosphyrus group in the Oriental faunal Region. Anopheles balabacensis is the primary vector of human malaria parasites in Thailand.

During the reporting period, at least 7 species of Aedes, Anopheles and Culex previously unreported from Thailand, were collected and reared. A probable new species of Culex was also collected. These specimens are undergoing further study in preparation for publication.

Reports of Anopheles campestris, a suspected vector of malaria in Thailand, and the collection of campestris - like adults, continue to occur in the Chiangmai Valley in northern Thailand. However, campestris may not be present in northern Thailand (36). A recent

collection of numerous reared barbirostris and "campestris" from Chiangmai is currently being prepared for study. Adult characters to separate these two species are known to be unreliable in Thailand, thus an examination of the pupal stage, which exhibits diagnostic characters, is necessary.

In Thailand, Anopheles philippinensis may be a secondary vector of human malaria pathogens (unconfirmed by dissection). Reid (37), however, demonstrated that philippinensis in Assam, Burma and Malaysia actually consists of 2 species, nivipes and philippinensis. Current studies at AFRIMS indicate that either Reid's division of philippinensis was not justified or that nivipes is the common species in Thailand and philippinensis is either absent or extremely rare. These studies involve the examination of over 900 ♂ and ♀ progeny with associated immature skins from 54 wild collected females. Reid (37) did not study progeny, and indicated that the study of progeny would confirm or refute the existence of nivipes as a valid species.

A major revisionary study was initiated during this period on the Aedes (Finlaya) kochi group of species in Thailand and Malaysia. At least 4 species are known from the study area and one species under study, poicilius, is a confirmed vector of Wuchereria bancrofti parasites in the Philippines. This group is found primarily in southern Thailand and extends as far north as Kanchanaburi Province. Adults feed readily on man and the immature stages are found primarily in Pandanus axils. To date, 2,040 adults and 1,668 slides have been examined and illustrations of the larval and pupal stages have been prepared. Detailed descriptions are still in preparation.

Manuscripts near completion or completed during the period include: Illustrated keys to the adult domestic mosquitoes of Thailand; Illustrated keys to the genera of adult and larval mosquitoes in Thailand; and The Myzomyia series of Anopheles (Cellia) in Thailand, with emphasis on intra-interspecific variations (Diptera: Culicidae).

6. Survey of Sylvatic Rodents for Serological Evidence of Rabies Virus Infection

OBJECTIVE: To capture sylvatic rodents in selected forested areas of Thailand and to test their serum for rabies neutralizing antibodies.

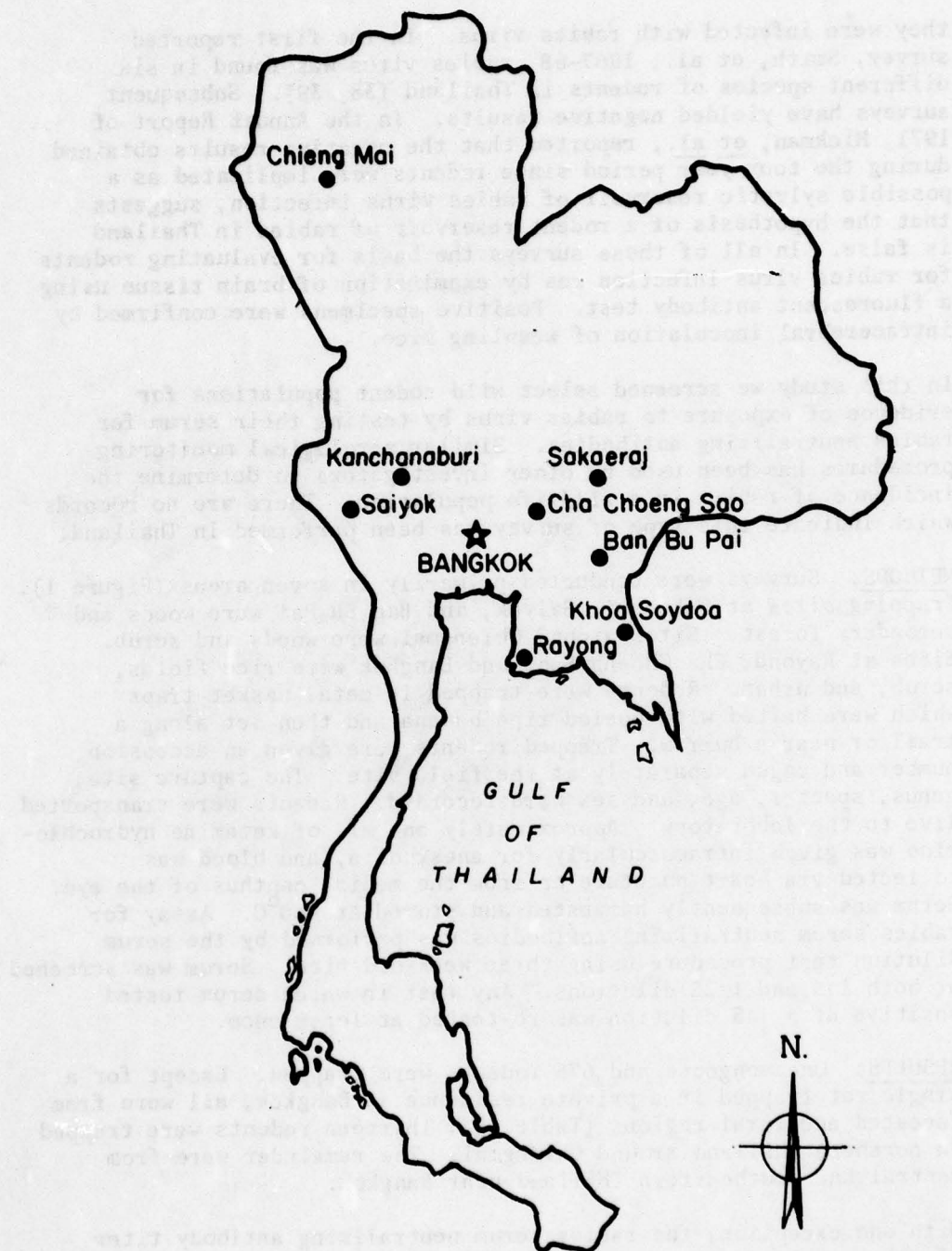
BACKGROUND: On several occasions investigators at SEATO Medical Research Laboratory have examined wild rodents to determine if

Table 18.

Listing of Animals Trapped For Serology Survey in 1977-78

Trapping Site	Sakaera]	Chieng Mai	Sai Yok	Rayong	Kanchana- buri	Ban Bu	Bangkok	Cha Cherng Sao	Total
Month	Jul-Aug 77	Sep 77	Oct 77	Nov 77	Nov 77	Dec 77	Dec 77	Jan 78	
<u>Rattus rattus</u>	24		8	26	10	16	-	55	139
<u>Rattus koratensis</u>	1	-	-	-	-	-	-	-	1
<u>Tupaia glis</u>	18	1	4	-	2	5	-	-	30
<u>Menetes berdmorei</u>	1	4	8	-	5	1	-	-	19
<u>Rattus sabanus</u>	6	-	-	-	-	17	-	-	23
<u>Rattus surifer</u>	77	-	6	-	4	63	-	-	150
<u>Bandicota indica</u>	-	5	-	51	1	-	-	219	276
<u>Rattus bukit</u>	-	1	-	-	-	-	-	-	1
Mongoose (<u>Herpestes javanicus</u>)	1	-	-	-	-	-	-	-	1
<u>Rattus exulans</u>	-	1	-	-	-	-	-	-	1
<u>Rattus berdmorei</u>	-	-	-	2	1	-	-	-	3
<u>Rattus rapit</u>	8	-	1	-	-	1	-	-	10
<u>Rattus norvegicus</u>	-	-	-	-	-	-	1	-	1
<u>Rattus losea</u>	-	-	-	-	-	4	-	-	4
<u>Bandicota savilei</u>	-	-	7	-	10	-	-	-	17
TOTAL	135	13	34	79	33	107	1	274	676

Figure 1



they were infected with rabies virus. In the first reported survey, Smith, et al., 1967-68, rabies virus was found in six different species of rodents in Thailand (38, 39). Subsequent surveys have yielded negative results. In the Annual Report of 1971, Hickman, et al., reported that the negative results obtained during the four year period since rodents were implicated as a possible sylvatic reservoir of rabies virus infection, suggests that the hypothesis of a rodent reservoir of rabies in Thailand is false. In all of these surveys the basis for evaluating rodents for rabies virus infection was by examination of brain tissue using a fluorescent antibody test. Positive specimens were confirmed by intracerebral inoculation of weanling mice.

In this study we screened select wild rodent populations for evidence of exposure to rabies virus by testing their serum for rabies neutralizing antibodies. Similar serological monitoring procedures has been used by other investigators to determine the incidence of rabies in a wildlife population. There are no records which indicate this type of survey has been performed in Thailand.

METHODS: Surveys were conducted primarily in seven areas (Figure 1). Trapping sites at Sakaeraj, Saiyok, and Ban Bu Pai were woods and secondary forest. Sites around Chiangmai were woods and scrub. Sites at Rayong, Cha Choeng Sao, and Bangkok were rice fields, scrub, and urban. Rodents were trapped in metal basket traps which were baited with peeled ripe banana and then set along a trail or near a burrow. Trapped rodents were given an accession number and caged separately at the field site. The capture site, genus, species, age, and sex were recorded. Rodents were transported live to the laboratory. Approximately one ml. of ketamine hydrochloride was given intramuscularly for anesthesia, and blood was collected via heart puncture or from the medial canthus of the eye. Serum was subsequently harvested and stored at -60°C . Assay for rabies serum neutralizing antibodies was performed by the serum dilution test procedure using three week old mice. Serum was screened at both 1:5 and 1:25 dilutions. Any test in which serum tested positive at a 1:5 dilution was re-tested at least once.

RESULTS: One mongoose and 675 rodents were trapped. Except for a single rat trapped in a private residence in Bangkok, all were from forested and rural regions (Table 18). Thirteen rodents were trapped in northern Thailand around Chiangmai. The remainder were from central and southeastern Thailand near Bangkok.

With one exception, the rabies serum neutralizing antibody titer of all animals was less than 1:5. The single exception was an adult R. surifer. Both a serum collected shortly after capture

22 December, and a second serum collected 24 January was tested. In two separate tests, the serum first collected had a titer of 1:5 and 1:8, respectively, and the later serum had a titer of 1:8. On the basis of FA testing and mouse inoculation, rabies virus could not be demonstrated in brain tissue of the R. surifer.

Since the first reports of rodent rabies in 1966-69, brain tissue has been examined from not less than 1,217 rodents. Rabies virus has not been detected in a single instance. Test procedures used for virus detection have been uniform throughout the entire survey period. Confirmation of laboratory findings obtained in 1966-69 is not possible because both the original brain tissue and the virus isolates have been lost.

7. Survey of Sylvatic Rodents for Serological Evidence of Leptospirosis Infection

OBJECTIVE: To determine the incidence of infection of Leptospirosis among sylvatic rodents in the area around Pakchong, Thailand.

BACKGROUND: Leptospirosis is known to infect many species of animals and man. One major source of infection of leptospirosis in human is known to be wild rodents.

The investigators joined the Scrub Typhus rodent trapping team from the Department of Medical Entomology and used the specimens trapped by this team in conducting this survey. Rodents were trapped in the vicinity of Special Forces camp Number 9, Pakchong, Nakorn Ratchasima. Once the Scrub Typhus Team had extracted their samples from the rodents, the rodents were turned over to the investigators for examination for serological evidence of leptospirosis infection.

METHODS: Blood for serologic analysis was obtained from each trapped rodent by either ocular bleeding or heart bleeding. Approximately 0.5 ml. of whole blood was placed on a strip of Whatman No. 4 filter paper and allowed to dry for one hour at room temperature. The agglutination (lysis) test for the presence of leptospirosis antibodies was performed on each of the blood specimens at the laboratory of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. This method has been previously described (40).

RESULTS: Results of the serological test for the presence of leptospiral antibodies in the 146 rodents trapped during this survey were negative. It should be noted that the agglutination (lysis)

test was performed at a dilution of 1:100 only. This survey concludes the study of the incidence of Leptospirosis in sylvatic rodents in the Pak Chong area.

8. Survey on the Incidence of Angiostrongylus sp. in Wild Rodents

OBJECTIVE: To determine the incidence of Angiostrongylus sp. in wild rodents in the area of Pakchong, Thailand.

BACKGROUND: Angiostrongylus sp., the rat lung-worm, has been shown to cause eosinophilic meningitis in man (41, 42, 43). Numerous cases of eosinophilic meningitis caused by Angiostrongylus sp. have been reported from Thailand (44). One case of human ocular angiostrongyliasis has also been reported from Thailand (45).

The investigators joined the Scrub Typhus Rodent Trapping Team from the Department of Medical Entomology and used the specimens trapped by this team in conducting this survey. One the Scrub Typhus Team had extracted their samples from the wild trapped rodents, the rodents were turned over to the investigators for examination for the presence of Angiostrongylus sp.

METHODS: A complete gross necropsy was performed on each rodent in the field. Particular attention was given to the gross examination of the lungs, mesenteric vessels, heart and liver of each specimen. Tissue from all organs was harvested and placed in 10% buffered formalin for histopathologic examination in the laboratory.

All nematodes found at necropsy were placed in alcohol-formol-acetic fixative for preservation and transport to the laboratory where definitive identification was carried out (46). Tissues were processed in the routine manner and examined microscopically for the presence of nematode parasites. Gross nematode parasites were given to Dr. Manoon, Faculty of Tropical Medicine, Mahidol University for identification.

RESULTS: The number and anatomic location of adult and/or larval helminths found at gross necropsy is given in Table 19. A total of 32 animals were found to be infested with internal parasites. Of these specimens collected and submitted for identification, only two were positively identified as Angiostrongylus cantonesis. Both A. cantonesis were found in Rattus rattus. One was located in the right atrium and the other was present in the pulmonary vein.

Table 19.

Anatomic Location of Helminth Parasite	Number of Animals
GI tract	14
Respiratory tract	5
Peritoneal cavity	7
Heart	1
Liver	1
Subcutaneous tissue	2
Muscle	1
Lymph node	1

A variety of other gross liver lesions were noted during necropsy of the rodents. These lesions ranged from cysts of various sizes caused by the larval tapeworm Cysticercus fasciolaris which occurred in 33 animals to white spots in the liver which were probably old migratory tracts caused by other parasites. A few other unremarkable incidental lesions were seen grossly.

The results of this rodent survey indicated an incidence of infection of Angiostrongylus cantonesis of 1.37% in wild rodents. This infectivity rate is slightly lower but compares favorably with the infectivity rate reported from this section of Thailand by others (41). This survey is complete and this report is the final one.

9. A Survey for Viral Agents Transmitted by Culicoides in Northern Thailand

OBJECTIVE: To isolate and identify viral disease agents from Culicoides collected in Chiangmai Valley.

BACKGROUND: As previously reported (47), collections of blood sucking midges of the genus Culicoides were made in rural villages of the Chiangmai Valley between March and September 1976, with the goal of isolating and identifying arboviruses from pools of midges. This report concludes the study.

METHODS: Pools of Culicoides mcDowelli were ground in 1.0 ml of M-199 with 5 percent fetal calf serum and antibiotics. 0.3 ml aliquots of the pool suspensions were assayed for plaque forming agents in LLC-Mk₂ cells by the direct and delayed plaque techniques.

RESULTS: Twelve pools of Culicoides mcDowelli with 76 to 277 midges per pool were assayed. No plaque forming agents were isolated.

10. Isolation of Dengue Viruses from Patients in Provincial Hospitals

OBJECTIVE: To isolate dengue viruses from dengue hemorrhagic fever patients admitted to provincial hospitals of Thailand and to compare the virus serotypes with those isolated in Bangkok.

BACKGROUND: Since 1962 the personnel of the Medical Research Laboratory, Armed Forces Research Institute of Medical Sciences, have isolated dengue viruses from patients of the Children's Hospital, Bangkok. During this period the dengue virus serotypes that were isolated varied considerably. Dengue-2 virus was isolated from patients in all years that isolations were attempted. In the

Table 20 Virus Isolations from Patients Admitted to Provincial Hospital (Aug-Oct 77) and Bangkok Children's Hospital (Jan-Dec 77) with the Diagnosis of DHF

Place	No. Studied	Confirmed DHF*	Probable DHF**	No. of Isolations	Identification					Unidentified
					D-1	D-2	D-3	D-4	CHIK	
Khon Kaen	42	31	1	14	0	1	6	6	0	1
Phrae	22	10	1	6	0	0	2	1	1	2
Ubol	35	33	1	5	0	1	3	0	0	1
Udorn	40	35	2	11	0	7	3	1	0	0
Provincial Hospitals Total	139	109	5	36	0	9	14	8	1	4
Children's Hospital (Bangkok)	493	233	103	82	0	37	11	19	2	13

* Diagnosis confirmed by HI serology

** No convalescent serum specimen

early 1960's, dengue-1, 3 and 4 were also present. Dengue-4 was isolated in 1965, but from 1969 through 1975 this serotype was not isolated from Bangkok patients (surveillance of dengue virus types by isolation was not conducted in Bangkok during the period 1966-1968). Approximately 50% of the viruses isolated from 1972 through 1975, were dengue-1 and 3 and the remainder were dengue-2 viruses. In 1976 dengue-4 was isolated much more frequently than dengue-1 and 3 viruses. The incidence of human dengue virus infections in Thailand increased earlier than expected and to unprecedented levels during 1977. The increase may have been due to the re-introduction of dengue-4 virus.

The purpose of this study was to isolate dengue virus from patients admitted to provincial hospitals and to compare the virus serotypes to those isolated from patients admitted to the Children's Hospital, Bangkok.

METHODS: After consultation with the Director, Division of Epidemiology of the Ministry of Public Health, studies were conducted at the provincial hospitals at Khon Kaen, Phrae, Ubol, and Udorn. From August through October, 1977, blood specimens were obtained from clinically diagnosed DHF patients who had been ill for four days or less. A second specimen was collected from the same patients two to five days later. A short history and physical examination were recorded for each patient.

Serum and plasma specimens were stored and transported to AFRIMS in liquid nitrogen. Virus isolation attempts were carried out on acute plasma specimens using the direct and delayed plaque techniques on LLC-Mk₂ cells. Viruses were identified by the standard plaque reduction neutralization technique using prototype dengue virus anti-sera prepared in monkeys. Acute and convalescent sera were assayed for antibody by the hemagglutination inhibition (HI) technique.

RESULTS: Applying standard laboratory criteria for the serologic diagnosis of DHF (four fold titer rise with convalescent titer \geq 640 or fixed high titer $>$ 1280 by HI serology), 109 of the 139 clinical diagnoses of DHF in the provinces were confirmed; 15 were not confirmed; and 5 lacked a convalescent serum specimen. As shown in Table 20 thirty-six dengue virus strains were isolated from patients of four provincial hospitals. The results of dengue virus isolations from Bangkok Children's Hospital for the year 1977 are presented in the same table for comparison. Dengue virus serotypes 2, 3, and 4 were isolated; however, dengue-4 viruses were detected most frequently. Isolates of dengue-3 virus were relatively more common in specimens from the provinces than from Bangkok.

11. A Serological Survey for Togaviruses (Arboviruses)
in a Well Defined Rural Thai Population

OBJECTIVE: To study the seroepidemiology of Togavirus (arbovirus) infections in a well defined rural Thai population.

BACKGROUND: Dengue virus was first specifically indentified as a cause of illness in Thailand in the early 1950's. At that time, and for many years thereafter, it was felt that dengue infections were endemic only in the large cities. More recently, it has been recognized that dengue infections also occur in rural populations. However, little is known of the prevalence of arbovirus antibody in rural Thailand, as most studies done outside of cities involved areas of epidemic illness.

As sera were already being collected from village populations for malaria and hepatitis studies, a serological survey for arbovirus infection was included.

MATERIALS AND METHODS: The materials and methods for this study are outlined elsewhere in this report (Seroepidemiologic Survey of Hepatitis B Virus Infection in a Rural Thai Village). Serum was submitted for routine hemagglutination inhibition test using an alpha virus (arbovirus group A) antigen, specifically Chikungunya (Chik) and flavivirus (group B arbovirus) antigens, specifically types Dengue 1-4 (DEN 1-4) and Japanese encephalitis (JEV).

RESULTS: Of a total population of 993 people over the age of 1 year in the village, sera from 526 had been tested by the time of this report. Of these, 230 sera were obtained from people from age 1 to 10. This age group made up over 60% of the village population. The prevalence of antibody to Chikungunya antigen in the whole population was 19.2%; it was 6.1% in the tested children ten years old or less and rose to 54.5% by the 40-40 year age group, remaining at that level in the few older persons studied. The flavivirus antibody, on the other hand, rose rapidly, starting at 20% in the few one year olds bled but reaching 89.2% by the age of four years and remaining over 80% for all but one of the remaining age groups.

This data serves as a good comparison to that collected in Bangkok in 1962 and 1977 and illustrates that flavivirus transmission is much more rapid in rural environments than in Bangkok. The antibody prevalences to flaviviruses are very similar to those found in Phnompenh in 1974 (48).

Table 21 Prevalence of Togavirus Antibodies
in a Well Defined Thai Rural Population

Age	No. Tested	Alpha Virus [*] Antibody		Flavivirus ^{**} Antibody	
		No.	(%)	No.	(%)
1	5			1	(20.0)
2	15			4	(26.0)
3	23			6	(21.0)
4	37	2	(5.4)	33	(89.2)
5	21			17	(80.9)
6	35	2	(5.7)	29	(82.9)
7	35	4	(11.4)	30	(85.7)
8	24	1	(4.1)	17	(70.8)
9	24	3	(8.3)	20	(83.3)
10-14	54	13	(24.0)	47	(87.0)
15-19	52	6	(11.5)	45	(86.5)
20-29	92	22	(23.9)	85	(92.4)
30-39	55	20	(36.4)	54	(98.2)
40-49	33	18	(54.5)	33	(100.0)
50	22	12	(54.5)	20	(90.9)
	526	103	(19.6)	441	(83.8)

* Chikungunya

** Dengue 1-4 and Japanese encephalitis

Table 22 Association Between Previous Exposure to Hepatitis B Virus and Arboviral Agents

Evidence of HBV Exposure	Total Villages Tested	Evidence of Exposure to Arboviral Agent					
		Dengue		JEV		Group A	
		+	-	+	-	+	-
+	179	162	17	162	17	61	118
-	307	260	47	228	79	62	245
		$\chi^2_y=2.85$ N.S.		$\chi^2_y=17.79$ P < .0005		$\chi^2_y=10.81$ p < .002	

Table 23 Relative Risk of Concurrent Evidence of Japanese B Encephalitis Virus Exposure and Hepatitis B Virus Exposure

Age	Sex	Hepatitis Exposure	Group A		RR*
			JEV Exposure		
			+	-	
<10	Female	+	18	4	1.49
		-	46	38	
	Male	+	16	6	1.32
		-	42	34	
>10	Female	+	93	3	1.01
		-	115	5	
	Male	+	35	4	0.97
		-	25	2	

RR* for: all males 1.29 all <10 1.40
all female 1.19 all >10 1.00
all villagers 1.22

*RR = Relative risk

Table 24 Relative Risk of Concurrent Evidence of Group A Arbovirus Exposure and Hepatitis B Virus Exposure

Age	Sex	Hepatitis Exposure	Group A		RR*
			Arbovirus Exposure		
			+	-	
<10	Female	+	0	22	indeterminate
		-	5	79	
	Male	+	4	18	2.76
		-	5	71	
>10	Female	+	38	58	1.16
		-	41	79	
	Male	+	19	20	1.20
		-	11	16	

RR* for: all female 1.43 all <10 1.45
all males 2.43 all >10 1.19
all villagers 1.69

*RR = Relative risk

Table 21 presents the arboviral serology results of the sample of villagers tested. The prevalences of the various arboviral agents are significantly different from those found in urban Thai populations (49). Table 22 however, demonstrates the association between evidence of Hepatitis B virus exposure (HB Ag and/or anti HB_s) and the prevalence of evidence of previous arbovirus infection. The associations appear to be age and sex dependant. Children under the age of 10, especially females, represent the majority of the correlation between Hepatitis B virus and Japanese encephalitis virus infections (Table 23). Table 24 presents similar data for Group A arbovirus antibody. With alphaviruses, concurrent markers of previous infection in males are responsible for the increased correlation between the two agents. While the age difference still exists, it is not as large as that found for JEV. Possible explanations include:

1. Proportion of population susceptible to all infection diseases.
 - a. by constitution
 - b. by increased interaction with their environment.
2. Similar mechanisms of disease transmission
 - a. focus of disease by location
 - b. similar vector.
3. Interaction between antibody to all diseases
 - a. similar antibody induced by one disease
 - b. laboratory test not specific.

The collection of materials from the village of Tablan is finished. This report presents preliminary data as the laboratory tests are still underway.

12. Isolation of Dengue Viruses from Leukocytes and Plasma of Dengue Hemorrhagic Patients

OBJECTIVE: To continue studies on isolation of dengue viruses from dengue hemorrhagic fever (DHF) patients of the Children's Hospital; to determine if virus can be isolated from leukocytes during natural dengue infections and to identify the cells infected.

BACKGROUND: Previous studies have shown that dengue viruses can be isolated from leukocyte preparations taken from patients with

dengue hemorrhagic fever (50); these studies were extended.

With this report we adopt the convention of reporting all isolations for a single calendar year, in this case January 1st, 1977 to December 31st, 1977. This period of reporting encompasses a single "dengue season" and will considerably facilitate year to year comparisons of DHF epidemics. Approximately half of specimens included in this report were included in last year's report.

METHODS: Clinical histories and heparinized blood samples were obtained from patients admitted to Bangkok Children's Hospital. The first day of fever was defined as the first day of illness. A follow-up serum sample was requested approximately 15 days after admission.

Heparinized blood specimens were separated into plasma and cell fractions by centrifugation. The cell fraction was resuspended in a solution of Dextran T-250, the red cells were allowed to sediment, and subsequent slow centrifugation pelleted the leukocytes. Viruses were isolated from plasma or leukocyte populations using the direct or delayed plaque method on LLC-Mk₂ cells.

Characterization of infected cells as "adherent" or "non-adherent" was accomplished by adding leukocyte suspensions to tissue culture flasks and incubating the cells for 2 hours at 37°C. Media and non-adherent cells were withdrawn, and the adherent cells in the flask washed once. LLC-Mk₂ cell suspensions were added to the flask, incubated overnight, and overlaid with agar. The non-adherent cells which had been withdrawn from the flask were mixed with a suspension of LLC-MK₂ cells, put in a second flask, incubated overnight and overlaid with agar.

RESULTS: A total of 81 viruses were isolated from the plasma and/or leukocytes of 336 patients hospitalized with hemorrhagic fever during 1977 (Table 25, Chikungunya virus was isolated from two patients; one was obtained from plasma while the other was isolated from leukocytes obtained from blood drawn on the eighth day of illness. The dengue virus isolates consisted of 67 strains which could be classified as Den-2, 3 or 4 and 12 other viruses that could not be identified. The latter viruses were considered to be dengue, as the serum of the patients from which these viruses were isolated showed a significant rise in dengue HI antibody titers. None of the dengue viruses were isolated from plasma alone, 14 from plasma and leukocytes and 56 from leukocytes alone (Table 26). Viruses were not isolated from plasma specimens with HI antibody titers

Table 25 Virus Isolations from Plasma and/or Leukocytes

Virus	Isolation	
	No.	%
Chikungunya	2	2.5
Dengue-1	0	0.0
Dengue-2	37	45.7
Dengue-3	10	12.3
Dengue-4	20	24.7
Dengue-?	12	14.8
Total	81	100

Table 26 Dengue Virus Isolations from Plasma and/or Leukocytes

Specimen	Isolations	
	No.	%
Plasma only	9	11.4
Leukocytes only	56	70.9
Both Plasma and Leukocytes	14	17.7
Total	79	100

Table 27 Identified Dengue Virus Isolations from Plasma and Leukocytes Tabulated against the Patients Homologous HI Titers

Homologous ¹ Titers	Isolations			
	Plasma	Leukocyte	Both	Total
10	-	2	2	4
20	-	4	4	8
40	2	4	4	10
80	1	6	-	7
160	-	7	3	10
320	-	5	-	5
640	-	3	1	4
1280	3	4	-	7
2560	2	3	-	5
5120	-	2	-	2
10240	-	5	-	5
Totals	8	45	14	67

¹ Reciprocal HI antibody titer

Table 28 Isolation of Dengue Viruses from Plasma and Leukocytes of Dengue Hemorrhagic Fever Patients.

Day of Disease	Total Studied	Virus Isolation			Total
		Plasma	Leukocytes	Both	
2	4	-	3	1	4
3	25	1	4	5	10
4	76	3	17	6	26
5	93	2	22	1	25
6	58	1	4	-	5
7	44	1	4	1	6
8-10	36	1	2	-	3
Total	336	9	56	14	79

Table 29 Isolation of Dengue Viruses from Adherent and Non-Adherent Leukocytes.

Leukocytes	Isolation	
	No.	%
Adherent cells only	11	52.4
Non-Adherent cells only	3	14.3
Both Adherent and Non-Adherent cells	7	33.3
Total	21	100

against homologous antigens greater than 1:2560; however, viruses were isolated from the peripheral blood leukocytes (PBL) of patients with homologous HI antibody titers of 1:10240 or greater (Table 27).

Most dengue virus isolations were obtained from plasma and leukocytes during the first five days of illness (Table 28), and the majority of the isolates were obtained during the febrile period.

Direct plaquing methods showed a range of 2 to 100 infectious centers per 3×10^5 leukocytes. The small number of infectious centers indicated that relatively few of the leukocytes produced infection in LLC-Mk₂ cell cultures. In initial trials to ascertain the type of leukocytes infected, virus isolations were attempted from cells adherent to plastic tissue culture flasks and from non-adherent cells. Dengue viruses were isolated from twenty-one sets of adherent and non-adherent cell populations (Table 29). Eleven sets yielded virus from adherent cells only, 3 sets from non-adherent cells and 7 sets from both adherent and non-adherent cells. Investigations are underway to further characterize the infected cell populations.

13. Dengue-2 Candidate Vaccine Studies: Challenge of Immunized Monkeys with Southeast Asian Wild Type Viruses

OBJECTIVE: To challenge monkeys immunized with Dengue-2 candidate vaccine with local wild type dengue strains.

BACKGROUND: Ten Indian Rhesus monkeys (*Macaca mulatta*) were immunized (17 Jan 1977) with a candidate Dengue-2 live attenuated virus vaccine strains (PR-159 (S1) Lot. No. 1 June 1976). (See Annual Progress Report 1976-1977). None of the ten monkeys developed detectable viremia. Four of these monkeys developed hemagglutination inhibition antibodies and six developed neutralizing antibodies against dengue-2 virus (KS-2472, MK2-4) by 30 days following vaccine administration. Of the six that developed antibody, three had neutralizing titers of 1:40 while three were 1:20. Five monkeys were inoculated with the Dengue-2 vaccine parent strain (PR-159, GM-6). All of these monkeys had proven viremia for three or more days (from the 2nd to the 8th day) and developed hemagglutination inhibition and neutralizing antibody (GMT's 1:210 and L:600 respectively) by the 30th day following inoculation. The purpose of this experiment was to determine if the immunized monkeys were protected against infection with wild type dengue strains present in Southeast Asia.

METHODS: The ten monkeys previously immunized with the candidate 2 vaccine strains were divided into three groups:

Group 1: This group consisted of two monkeys without neutralizing antibody (E290, E293) and another two with neutralizing antibody (E231, E299) (Table 30). This group received a wild type dengue-2 strain (BM50-76, MK₂-2) which had been isolated from a mosquito collected from the home of a patient with dengue hemorrhagic fever and passed twice in LLC-Mk₂ cells. On initial isolation both large and small plaques were seen. The virus was administered in a dose of 0.5 ml containing 1.1×10^5 PFU.

Group 2: Two vaccine immunized monkeys (F15, E295) and two monkeys (F17, F66) immunized with parent strain of dengue-2 virus (PR-159, GM-6) were included in this group (Table 31). These monkeys received a wild type dengue-3 strain (CH-1337-74, MK₂-10) with a titer of 2.3×10^4 PFU/0.5 ml. This strain was isolated from the serum of a dengue hemorrhagic fever patient and passed 10 times in LLC-Mk₂ cells.

Group 3: This group included 2 vaccine immunized monkeys without neutralizing antibody (E294, E298) and 2 vaccine immunized monkeys with low (E229) and high (E301) neutralizing antibody titers (Table 32). These monkeys received a booster with 3.3×10^2 PFU of candidate vaccine PR-159 (S1) lot. 1.

All monkeys were inoculated subcutaneously with 0.5 ml of the appointed strain of virus. The monkeys were examined the day before inoculation and daily throughout the course of the experiments.

The following blood specimens were obtained on day 0 prior to the inoculation and on days 1-10, 15, and 30 following immunizations: EDTA blood for hematology including hemoglobin, hematocrit, white blood cell and platelet count; serum for serological tests and SGPT; and heparinized blood for virus isolation from plasma and blood leukocytes. Virus isolation was performed by standard direct and delayed plaque technique in LLC-Mk₂ and by the mosquito-inoculation technique.

RESULTS: Wild type dengue-2 challenges of immunized monkeys:
The first group of monkeys (E290, 231, and 299) that were previously immunized with candidate dengue-2 vaccine were challenged with wild type dengue-2 strain (BM50-76, MK₂-2) 4 months following the initial immunization. Viremia occurred in the three out of four monkeys (Table 30) (those three in which the neutralizing antibody titer had fallen to $\leq 1:100$ by the time of challenge). Viremia began on the third day following inoculation and lasted for 2 days. The monkey (E299) with a neutralizing titer of 1:100 in the

Table 30 Viremia and Antibody Responses of Dengue-2 Wild Type Challenge of Rhesus Monkeys After Immunization with Candidate Dengue-2 Vaccine.

Monkey Number	Original Inoculum (PFU)	Neutralize Titer at Day 30	Challenge Inoculum (PFU)	Viremia Days		Days Post Challenge	Reciprocal Antibody Titers											
							HI			CF			NT			D-1	D-2	D-3
				Plasma	LK		D-1	D-2	D-3	D-4	D-1	D-2	D-3	D-4	D-1	D-2	D-3	D-4
E-290	Vaccine (3.3x10 ⁶)	0	BM-50-76 (1.1x10 ⁵)	4,5,4,5	None	d-0 d-15 d-30	0** 1280 640	0 5120 640	0 2560 640	0 5120 1280	0 ND ND	0 256 128	0 ND ND	0 ND ND	0 10 10	0 640 640	0 10 10	0 160 160
E-293	Vaccine (3.3x10 ²)	0	BM-50-76 (1.1x10 ⁵)	4,5	None	d-0 d-15 d-30	0 640 320	0 1280 640	0 1280 640	0 1280 640	0 ND ND	0 128 128	0 ND ND	0 ND ND	0 10 10	0 160 160	0 10 10	0 100 40
E-231	Vaccine (3.3x10 ²)	5	BM-50-76 (1.1x10 ⁵)	3,4,5	None	d-0 d-15 d-30	0 640 160	0 1280 640	0 1280 320	0 1280 640	0 ND ND	0 256 128	0 ND ND	0 ND ND	0 ND ND	0 640 640	0 40 10	0 640 640
E-299	Vaccine (3.3x10 ²)	140	BM-50-76 (1.1x10 ⁵)	None	None	d-0 d-10 d-30	0 20 10	40 320 160	20 80 40	20 160 80	ND ND ND	0 128 128	0 ND ND	0 ND ND	ND ND ND	100 640 640	0 0 0	0 0 0

* Leukocytes

** Titer of "0" signifies <1:10 for HI, <1/4 for CF, and <1/10 for neutralizing antibody titers

*** Not Done

Table 3 | Viremia and Antibody Responses of Dengue-2 Immunized Monkeys Challenged with Dengue-3 Virus.

Monkey Number	Original Inoculum (PFU)	Neutralize Titer at Day 30	Challenge Inoculum (PFU)	Viremia Days Plasma LK*	Days Post Challenge	Reciprocal Antibody Titer											
						HI			CF			NT			D-1	D-2	D-3
						D-1	D-2	D-3	D-1	D-2	D-3	D-1	D-2	D-3			
E-15	Vaccing (3.3×10^2)	10	D-3 (2.3×10^4)	4, 5	d-0 d-15 d-30	0 320 160	0 320 160	0 1280 640	0 1280 640	0 1280 640	0 1280 640	ND ND ND	ND ND ND	ND ND ND	0 10 0	10 320 550	10 80 320
E-297	Vaccing (3.3×10^2)	80	D-3 (2.3×10^4)	4, 5	d-0 d-15 d-30	0 320 320	0 640 320	0 1280 640	0 1280 640	0 1280 640	0 1280 640	ND ND ND	ND ND ND	ND ND ND	0 0 0	0 250 320	0 80 80
F-17	D-2 Parent (1.3×10^5)	222	D-3 (2.3×10^4)	4, 5	d-0 d-15 d-30	20 2560 1280	160 1280 1280	40 2560 1280	40 5120 2560	40 5120 2560	40 5120 2560	ND ND ND	ND ND ND	ND ND ND	0 300 300	0 340 190	0 160 320
F-66	D-2 Parent (1.3×10^5)	680	D-3 (2.3×10^4)	4, 5, 6	d-0 d-15 d-30	160 5120 5120	160 5120 2560	160 5120 5120	320 5120 2560	320 5120 2560	320 5120 2560	ND ND ND	ND ND ND	ND ND ND	0 640 640	0 640 640	0 160 320

* Leukocytes
** Not Done

Table 32 Viremia and Antibody Response of Rhesus Monkeys Following Booster Immunization at 126 Days After Primary Immunization.

Monkey Number	Original Inoculum (PFU)	Neutralize Titer at Day 30	Challenge Inoculum (PFU)	Viremia Days		Days Post Challenge	Reciprocal Antibody Titers											
				Plasma	LK*		HI			CF			NT					
							D-1	D-2	D-3	D-4	D-1	D-2	D-3	D-4	D-1	D-2	D-3	D-4
E-294	Vaccine (3.3x10 ²)	0	Vaccine (2.5x10 ³)	None	None	d-0 d-15 d-30	0 20 10	10 80 80	0 40 40	0 40 20	ND** ND ND	4 16 8	ND ND ND	ND ND ND	0 0 0	0 90 170	0 0 0	0 0 0
E-298	Vaccine (3.3x10 ²)	0	Vaccine (2.5x10 ³)	None	None	d-0 d-15 d-30	0 20 10	10 160 80	0 40 20	0 80 40	ND ND ND	2 16 32	ND ND ND	ND ND ND	ND ND ND	0 300 160	0 10 10	0 10 10
E-228	Vaccine (3.3x10 ²)	20	Vaccine (2.5x10 ³)	None	None	d-0 d-15 d-30	10 40 40	40 160 80	20 80 40	12 80 80	ND ND ND	2 64 32	ND ND ND	ND ND ND	0 ND ND	0 200 250	0 10 10	0 360 360
E-301	Vaccine (3.3x10 ²)	53	Vaccine (2.5x10 ³)	None	None	d-0 d-15 d-30	0 20 10	0 80 40	0 20 10	0 40 20	ND ND ND	2 32 16	ND ND ND	ND ND ND	ND ND ND	0 160 160	0 0 0	0 0 0

* Leukocytes
** Not Done

Table 33 Dengue-2 Challenge of Rhesus Monkeys After Booster Immunization with Candidate Dengue-2 Vaccine*

Monkey Number	Viremia	Reciprocal Antibody Titers vs Dengue-2									
		CF			HI			NT			
		Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	
E-294	None	4	256	512	0	>10240	1280	60	>640	>640	
E-298	None	8	256	256	40	>10240	2560	80	>640	>640	
E-301	None	10	512	256	0	1280	1280	80	>640	>640	

* Inoculum per monkey was 2×10^6 PFU of wild type dengue virus, MB-50-76.

prechallenge blood did not experience viremia. All monkeys developed high titers of neutralizing, hemagglutination inhibiting, and complement fixing antibodies (Table 30) in response to the challenge.

Wild type dengue-3 challenges of immunized monkeys: All monkeys in the second group developed viremia following dengue-3 challenge, and all developed a response of HI, CF, and NT antibodies (Table 31).

Reimmunization with candidate dengue-2 vaccine: In the third group, following booster immunization with dengue-2 candidate vaccine, no viremia was detected, but all monkeys showed CF, HI, and N. antibody responses to dengue-2 (Table 32).

Challenge with wild type dengue-2 following booster immunizations: Three monkeys which has received the booster immunization were challenged approximately 10 months later with 2×10^6 PFU of wild type dengue-2 BMSO-76 (Table 33). No viremia was documented in any of the challenged monkeys and again all redeveloped high titers of CF, HI, and N antibodies.

14. Isolation of Influenza Viruses During the 1977-1978 Epidemic

OBJECTIVE: To define the etiologic agent of the 1977-1978 influenza outbreak in Thailand.

BACKGROUND: Since the pandemic of 1968 (51, 52) influenza has not been a major public health problem in Southeast Asia. Only minor outbreaks have been reported in rural residents (53, 54) and in an isolated hill tribe of northeast Thailand (55). This report summarizes observations made during an influenza outbreak in Bangkok and surrounding provinces in January and February, 1978.

METHODS: Throat washings were obtained from patients with acute influenza-like syndromes. Specimens were treated with an anti-bacterial and antimycotic solution, and inoculated into the amniotic sac of nine to ten days old embryonated chicken eggs. Amniotic and allantoic fluid were harvested 48 to 72 hours later. The presence of hemagglutinating virus was detected using 0.5% chicken red blood cells. Identification of influenza virus isolates was performed by hemagglutination inhibition tests employing reference anti-sera prepared for prototype influenza strains.

Table 34. Hemagglutination Inhibition Test for Identification of Influenza Isolates 1978

Antiserum											
Antigen	A/FM/1/47/H1N1	A/NJ/8/76	A/TEX/1/77	B/HK/5/72	A/HK/68	A/JAP/305/57	A/BK/1/78(H1N1)	Booster antiserum	A/USSR/90/77/	(H1N1)	
A/FM/1/47 (H1N1)	640	<10	<10	<10	<10	<10	<10	640	320		
A/NJ/8/76	<10	160	<10	<10	<10	<10	<10	40	<10		
A/TEX/1/77	<10	<10	320	<10	10	<10	<10	<10	<10		
B/HK/5/72	<10	<10	<10	320	<10	<10	<10	<10	<10		
A/HK/68	<10	<10	20	<10	640	<10	<10	10	<10		
A/JAP/305/57	<10	<10	<10	<10	<10	160	<10	<10	<10		
A/BKK/1/78 (AFRIMS/001/78)	160	<10	<10	<10	<10	<10	<10	640	320		
A/USSR/90/77 (H1N1)	160	<10	<10	<10	<10	<10	<10	640	320		
A/BKK/2/78 (AFRIMS/016/78)	160	<10	40	<10	<10	<10	<10	1280	640		
A/BKK/3/78 (AFRIMS/038/78)	160	<10	<10	<10	<10	<10	<10	640	320		
A/BKK/4/78 (AFRIMS/082/78)	160	<10	<10	<10	<10	<10	<10	640	320		
A/BKK/5/78 (AFRIMS/083/78)	80	<20	<20	<20	<10	<10	<10	1280	640		
A/BKK/6/78 (AFRIMS/107/78)	40	<20	<20	<20	-	<10	<10	640	320		
A/BKK/7/78 (AFRIMS/063/78)	160	<20	40	<20	-	-	-	320	320		
A/BKK/8/78 (AFRIMS/053/78)	40	<20	<20	<20	-	-	-	1280	640		

RESULTS: Sporadic clinical cases of influenza were observed in Bangkok in early January 1978. During this month three of four specimens submitted from Children's Hospital were positive for influenza. These isolates were closely related to influenza A/FM/1/47 (H1N1), which had recently been reported to be causing an outbreak in Moscow. A wide outbreak began in Bangkok in early February 1978 and affected mostly young adults. Fifty-six throat washings were collected from children at four Bangkok schools with typical influenza-like syndromes. Fifteen hemagglutinating agents were isolated. In a city near Bangkok, Prachinburi, an outbreak occurred in late February. Four of 14 throat washing from ill school children were positive.

The overall rate of isolation was 22 out of 74 cases. All isolates were shown to be closely related to A/FM/1/47 and A/USSR/90/77 (H1N1) (Table 34). All isolates were from children and young adults ages from 8 to 20 years old; however, clinical illness was rare in adults and therefore no specimens were obtained from adults. Isolates were forward to Walter Reed Army Institute of Research, to the Center for Disease Control in Atlanta and to the WHO reference center in London for confirmation.

15. Ecology and Epidemiology Studies of Dengue Viruses in Din Daeng, Bangkok, Thailand

OBJECTIVES:

1. To determine the seasonal incidence of apparent and inapparent dengue virus infections in Bangkok adults and children.
2. To establish the duration and magnitude of the antibody response to primary and secondary arbovirus infection, including dengue viruses type 1, 2, 3, 4 and Japanese encephalitis (JE) virus.
3. To assess experimentally the ability of wild A. aegypti to serve as a vector of dengue viruses on a seasonal basis.
4. To determine the seasonal prevalence rate of dengue viruses in adult and immature A. aegypti populations.
5. To determine the population density of the wild A. aegypti population on a seasonal basis and seasonal availability and utilization of artificial containers by this species for oviposition.

BACKGROUND: Epidemiological and ecological investigations have shown that dengue viruses are endemic in Bangkok and that the primary vector and vertebrate host are Aedes aegypti and man, respectively (56, 57). Apparent human infections occur throughout the year. However, a marked increase in the incidence of illness has been observed during the rainy season (June-September), and age specific attack rate data have placed children 15 years of age or less at greatest risk (57). Early findings indicated that the variation in the incidence of infection was related to the population density of A. aegypti (58). More recent data suggest that the magnitude of change in absolute population size of A. aegypti in Bangkok was not large enough to explain the seasonal fluctuations in the incidence of apparent dengue virus infection (59). The latter investigation was extended to consider the longevity and blood feeding patterns of A. aegypti. Data did not show seasonal variation in 24 hour survival; however, biting rates appeared to vary as indicated by a marked decrease during the cool season of the year (60). According to the authors, the decrease in biting rates and a possible increase in the length of the extrinsic incubation period of the vector may cause the decrease in the incidence of dengue virus infections in man during this part of the year.

Except for the rainy season, data relative to the seasonal prevalence rates of dengue viruses in the A. aegypti population of Bangkok is lacking. In addition, no consideration has been given to the vector competence of this species during the different seasons of the year. Available data have shown that temperature affected the degree of susceptibility of mosquito to infection with arboviruses and the time required before transmission of arbovirus was accomplished by bite (61, 62). These factors and the possibility of seasonal changes in longevity, biting activity, etc. of A. aegypti may prove to influence the seasonal variation in prevalence of dengue viruses as well as the apparent variation of the circulation of different dengue virus serotypes in the mosquito and human population.

Transovarial transmission of arboviruses in their respective natural mosquito vector has been proven for some California encephalitis group viruses (63, 64). Recent experimental findings have shown that dengue viruses and JE virus can be transmitted transovarially by Aedes albopictus (65, 66). Evidence of transovarial transmission of dengue viruses in natural populations of mosquitoes has not been reported.

Estimates of A. aegypti population density employing human biting and landing counts, sweep nets, aspiration-vacuum sweep of resting adults, and dipping collections for larvae and pupae are biased by a number of individual human traits dependent on the collectors (67). Other collecting techniques, developed to avoid these problems include the pyrethrin knockdown, oviposition traps, and the one larvae per container techniques. The former test is biased by space, timing, and sealing problems, and a reluctance of the collectors to work in the presence of pyrethrin aerosol spray. The latter two techniques are better tools, but they cannot be employed alone for population density estimates.

METHODS: Study area: The study area for this investigation was a section of the Din Daeng area of Bangkok (Figure 2). The area is circumscribed by Prachasonkro Road on the north, Soi Charasongkhro on the west, Din Daeng I Road on the east and Klong Sam Sen on the south. Included in the area are 20 four-floor apartment buildings, many two-three storey shop-houses, approximately 4 acres of confluent single storey slum dwellings and a few single or two storey residential homes. The total human population of the study area was between 13,000 to 14,000.

Census of the population and mapping of the area: One hundred families of the Din Daeng study area that had a child attending Philbuonprachasan School were randomly selected for the study. Each family was interviewed regarding address, income level, occupation, family size, ages, general health, and cultural and behavioral practices possibly related to disease recognition. Interviews of the families and the mosquito habitat surveys were conducted concurrently and on a seasonal basis. The entire area was mapped in regard to layout of housing, streets and other permanent landmarks.

Dengue virus infection of the human population: The seasonal incidence of dengue, Chikungunya, and JE infections was based on seroconversion rates determined by hemagglutination inhibition tests (68). Blood was obtained from family members before and after each season of the year. Overt dengue virus infections were determined by bi-weekly visits to the residence of each family in the study population. Classification of apparent and inapparent infection was based on previously defined criteria (69, 70).

Seasonal assessment of the vectorial capacity of A. aegypti: A. aegypti larvae were collected from wild populations within the Din Daeng study area. Adult mosquitoes reared from the larvae

were allowed to ingest graded doses of dengue virus to establish the threshold level of infection. The extrinsic incubation period was determined by allowing individual mosquitoes to ingest normal blood from a hanging drop suspension at intervals after the mosquitoes ingested dengue viruses (71). An aliquot of the remaining blood suspension was assayed to determine if a particular mosquito secreted virus while feeding. Assay of blood and mosquitoes for virus was performed by plaque assay employing LLC-Mk₂ cells (72). Temperature and humidity were monitored continuously during the experiments and were maintained similar to that of the study area.

Seasonal dengue virus prevalence rates in *A. aegypti*: Adult *A. aegypti* and immature stages of this species will be assayed for virus by the mosquito inoculation technique employing *Toxorhynchites splendens* as a bioassay host (73).

Survey of mosquito larval habitats: The availability of *A. aegypti* larval habitats and the utilization of these sites by this species was determined during each season of the year. Residences of the 100 families in the study were surveyed indoors and outdoors to determine the total number of potential and positive larval breeding sites. A definition for containers inside or outside of houses was determined on the basis of a roof or roof-like structure above the container(s). Lids may occur on containers in both categories; however, if rainwater could flow or fall into the containers, then it was considered to be outside. Each container was searched thoroughly employing a flashlight and a 4 oz. suction syringe for *A. aegypti* larvae and pupae.

A standardized water container (ong jar) was placed in the residence of each of the 100 families selected for study. These jars will serve to provide estimates of the population density of *A. aegypti* obtained through concurrent employment of different, complementary sampling techniques. Each sampling device is being designed to focus on a certain life stage, behavioral factor, and/or physiological state of the mosquito population. The sampling methods (traps) described below are being designed to eliminate the bias of the human traits listed above and will be employed, provided preliminary tests show them to be reliable and effective sampling devices.

A floating larval trap will be used to determine the population density of *A. aegypti* larvae and pupae. These traps will be used on a rotating schedule throughout the 100 family units and collections will be made during one 24 hour period each week.

Emergence cone traps that fit over the mouth of water jars will be used to determine adult emergence patterns and densities during each season of the year. Gravid adults captured in these trap will be separated from the newly emerged mosquitoes and will be used for virus isolation studies.

An estimate of the oviposition rate of A. aegypti will be determined seasonally by estimating the number of gravid females. This will be determined by capturing gravid females in a specially designed trap that captures the female, but does not allow her to reach a substrate for oviposition. The gravid condition of trapped females will be confirmed by microscopic examination and gravid females will be saved for virus isolation attempts.

Additional sampling for estimating the adult density will be attempted by using a suction type trap. This trap will be designed to sample active flying adults, or by attracting them to a resting site. All females trapped by this method will be assayed for virus.

RESULTS: To assess the possible error because of biased sampling of the adult population of Din Daeng, (e.g. those adults who are always at home have a greater chance of being sampled) the serum specimens collected from adults in the Din Daeng area during the dry season survey were tested for arboviral antibody. Of 94 adults, ranging in age from 19 to 70, 92 (98%) had evidence of flavivirus infection and 74 (79%) had evidence of alphavirus infection. Because of these very high rates of seropositivity and a lack of male/female difference or age-dependent variation, the adult sample was considered adequate. All other antibody determinations are in progress.

The development of techniques for vector competence studies was hampered by the refusal of A. aegypti to ingest virus-blood suspension. Subsequent experiments in which the virus-blood suspensions were supplemented with 10% sucrose yielded a 90 to 100% feeding rate. Preliminary results indicated that this technique was effective for infecting colony A. aegypti with dengue virus types 1, 2 and 3. Ten to 20% of the mosquitoes became infected after ingesting $4.0 \log_{10}$ SMICLD₅₀/1.0 ml. of dengue virus type 1. After ingesting $6.0 \log_{10}$ SMICLD₅₀ of the same virus type, the infection rate increased to 60%. Approximately 20% of the A. aegypti became infected by employing a dose of $6.5 \log_{10}$ SMICLD₅₀ of dengue virus type 2. A similar dose of dengue virus type 3 yielded an infection rate of 60 to 90%. A. aegypti failed to become infected after ingesting $5.0 \log_{10}$ SMICLD₅₀ of dengue virus type 4. Attempts to demonstrate transmission of dengue viruses by infected A. aegypti were not successful.

Studies are in progress to assess the capability of A. aegypti of the Din Daeng study area to serve as a vector of dengue viruses during different seasons of the year and to correlate vector competence of A. aegypti populations with the genetic composition of this species. A description and the results of studies to develop techniques for establishing the genetics of A. aegypti populations are reported in the Entomology Section of this Annual Progress Report.

A. aegypti larvae were collected for transovarial transmission studies from the premises of 50 houses of dengue hemorrhagic fever patients in Bangkok. A total of 531 pools consisting of 13,005 larvae were collected from 1 November 1977 through 20 March 1978. Attempts to isolate dengue virus from 172 pools (4,280 larvae) were unsuccessful. Of the 172 pools, 100 were assayed by direct and delayed plaque technique and by the mosquito inoculation technique in conjunction with fluorescent antibody assay. The other pools were assayed by the latter technique. Attempts to isolate dengue viruses from two pools of 25 females and four pools of 42 male A. aegypti reared from field collected larvae were unsuccessful.

The number of A. aegypti larvae collected during each month and the average number of larvae obtained from each house is presented in Table 35. A. aegypti larvae were found in 76.6% (49/64) of the houses. Of 12,318 of the total 13,005 larvae collected, 32.8% and 67.2% were collected from containers located inside and outside of the house, respectively. A. aegypti larvae collected inside houses were obtained from water jars located in bathrooms (45%) water jars in kitchens (22%), water jars in bedrooms (13%) and the remainder were found in water jars in unspecified locations. Ninety-five percent of the larvae collected outside houses were found in water jars and 5% were obtained from claypots, tin cans, tires and ant-traps. The average number of A. aegypti collected per house was 203; however, the density of the larvae population was associated with a decrease in the number of larvae occurring in both inside and outside breeding containers. Containers of 20 houses were sampled a second time during January, February, and March 1978. A. aegypti larvae were present in all houses; however, the number of larvae collected was 1,768 compared to 3,706 larvae obtained during November and December, 1977. Virological and ecological investigations of A. aegypti larvae will continue but the studies will be conducted in Din Daeng study area as described in this report.

Investigations of the seasonal availability and utilization of artificial containers by A. aegypti in the Din Daeng study area began in April 1978. The residence of each of the 100 families was surveyed inside and outside during the dry season, 3-18 April,

and the early wet season, 30 May - 15 June. Natural containers were not found during either survey, as vegetation is practically non-existent in this densely packed urban area. The most common containers with water found in the residences were clay ceramic water (ong) jars, ant traps, flower vases, tin cans, plastic buckets and pans, and cement water basins. A seasonal comparison of the containers with water and those with A. aegypti is shown in Table 36. Initially, "containers with water" was considered a valuable index, however, the subjectiveness of deciding which container should be counted (numerous plastic wash basins, buckets, etc. that are used daily) has made accurate counts nearly impossible. Consequently, "containers with A. aegypti" is considered to be the most valuable index. As shown in Tables 36 to 38 there was a considerable increase in the number of A. aegypti positive containers, both inside and outside houses, between the dry season and the early wet season. In addition, houses positive for A. aegypti larvae increased from 24% during the dry season to 43% for the early wet season.

The seasonal prevalence of A. aegypti larvae and pupae inside and outside houses of different residential types is summarized in Tables 39 and 40. These data demonstrated an increase in A. aegypti positive containers both inside and outside of houses between the dry season and the early wet season. The only residential type not affected by the seasonal increase in water available to the family units was the high rise-flats for outside containers. Figure 3 depicts the monthly precipitation levels as recorded by the Bangkok Meteorological Station. The slum residences yielded the highest number of A. aegypti positive containers and family houses during both seasons. However, shop-house residences had the highest percentage of inside positivity for A. aegypti during the early wet season. These data suggest that the slum residences maintain the largest reservoir of A. aegypti through the dry season.

An inexpensive larval trap designed to float on the water surface in water jars has been developed and tested (Table 41). This circular trap is made of clear plastic and measures approximately 13 cm in diameter and 13-14 cm. in depth. The trap is designed such that larvae moving vertically toward the surface of the water are captured by passing through the aperture of the apex of a cone that leads into the collecting container. The data suggest that this trap is a highly efficient collector of A. aegypti and Culex quinquefasciatus larvae and/or pupae (Table 41 and 42). Although the trap is free of human collecting bias, the high returns in just 24 hours, suggest that the trap is biased, probably because larvae congregate around floating objects.

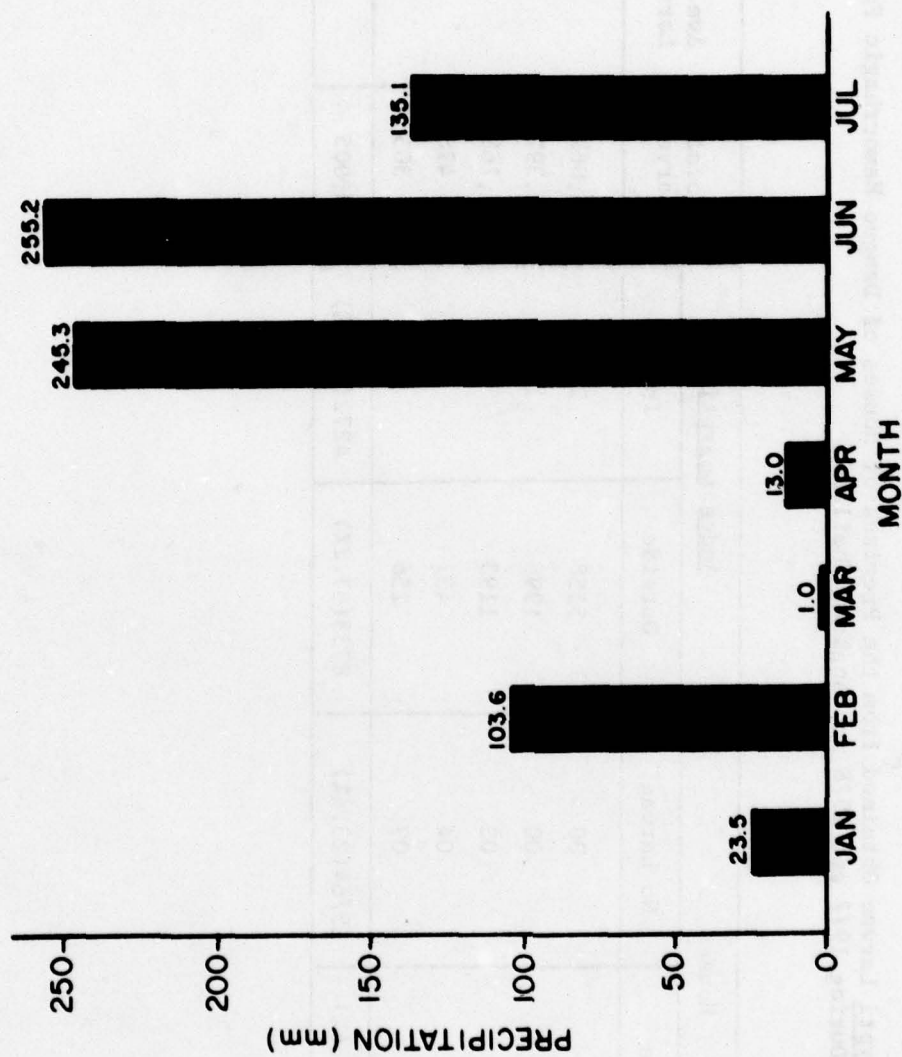


FIGURE 3. AMOUNT OF PRECIPITATION FOR JANUARY THROUGH JULY, 1978, BANGKOK, THAILAND.

Table 35 Aedes aegypti Larvae Obtained from the Premises of Houses of Dengue Hemorrhagic Fever Patients During 1977 and 1978 in Bangkok, Thailand

Date	Houses		<u>Aedes aegypti</u>		Total Larvae	Ave. Number Larvae/House
	Larvae	No Larvae	Outside	Inside		
Nov 77	10	00	5558	2504	8,062	806
Dec 77	10	00	1295	1097	2,392	239
Jan 78	16	02	1193	575	1,768	098
Feb 78	08	04	431	47	478	040
Mar 78	05	09	256	49	305	022
	49/64 (76.6%)	15/64 (23.4%)	8733 (67.2%)	4272 (32.8%)	13,005	203

Table 36 Seasonal Utilization of Containers for 100 Houses by Aedes aegypti Larvae and/or Pupae,
Din Daeng, Bangkok, Thailand

Date of Surveys	Containers with Water				Percentage of Containers with <u>A. aegypti</u>		Percentage of Houses Positive for <u>A. aegypti</u>
	Larvae and/or Pupae		No Larvae or Pupae				
	Inside	Outside	Inside	Outside	Inside	Outside	
3-18 April, 1978 (Dry Season)	55	11	532	68	9.4 (55/587)	13.9 (11/79)	29
30 May-15 June (Early Wet Season)	86	24	489	72	15.0 (86/575)	25.0 (24/96)	43

Table 37 Seasonal Change in Utilization of Containers Inside 100 Family Dwellings* by Aedes aegypti Larvae and/or Pupae, Din Daeng, Bangkok, Thailand

Date of Survey	<u>A. aegypti</u> Larvae and/or Pupae			% Change (+) from Dry Season		
	Containers	Dwellings	Container Dwelling	Containers	Dwellings	Container Dwelling
3-18 April 78 (Dry Season)	55	25	2.20	-	-	-
30 May-15 Jun 78 (Early Wet Season)	86	37	2.32	+56	+48	+5

* Same family dwellings used during both surveys

Table 38 Seasonal Change In Utilization of Containers Outside 100 Family Dwellings* by Aedes aegypti Larvae and/or Pupae, Din Daeng, Bangkok, Thailand

Date of Survey	<u>A. aegypti</u> Larvae and/or Pupae			% Change (+) from Dry Season		
	Containers	Dwellings	Container Dwelling	Containers	Dwellings	Container Dwelling
3-18 April, 78 (Dry Season)	11	8	1.37	-	-	-
30 May-15 Jun, 78 (Early Wet Season)	24	14	1.71	+118	+75	+25

* Same family dwellings used during both surveys

Table 39 The Seasonal Utilization of Containers Inside 100 Houses* by *Aedes aegypti* Larvae and/or Pupae for Different Types of Residences and the Number of Larvae and/or Pupae Pooled for Virus Isolation Studies

Residential Type	# of Units	Dry Season				Early Wet Season			
		Positive for <i>aegypti</i>			#Larvae & Pupae**	Positive for <i>aegypti</i>			#Larvae & Pupae Pooled
		Containers	Units	Cont./Unit		Containers	Units	Cont./Unit	
Highrise Flats	44	9	2	4.50	118	15	6	2.50	189
Slums	36	33	15	2.20	417	40	18	2.22	406
Shop or House	20	13	8	1.63	164	31	13	2.38	420
Total	100	55	25	2.20	699	86	37	2.32	1,015

* The same family units used during both surveys

** Larvae and pupae for virus isolation studies

Table 40 The Seasonal Utilization of Containers Outside 100 Houses* by *Aedes aegypti* Larvae and/or Pupae for Different Types of Residences and the Number of Larvae and/or Pupae Pooled for Virus Isolation Studies

Residential Type	# of Units	Dry Season				Early Wet Season			
		Positive for <i>aegypti</i>			#Larvae & Pupae**	Positive for <i>aegypti</i>			#Larvae & Pupae Pooled
		Containers	Units	Cont./Unit		Containers	Units	Cont./Unit	
Highrise Flats	44	0	0	0	0	0	0	0	0
Slums	36	10	7	1.43	153	19	12	1.58	224
Shop or House	20	1	1	1.00	20	5	2	2.50	70
Total	100	11	8	1.37	173	24	14	1.71	294

* The same family units used during both surveys.

** Larvae and pupae for virus isolation studies.

Table 41 Aedes aegypti Larvae Captured in Traps During a 24 Hour Period

Trials *	Date Test Ends	Larvae Trapped Per Container					Captured	
		Trap 1	Trap 2	Trap 3	Trap 4	Trap 5	Total	%
1	4 Aug	24	56	61	70	48	259	51.8
2	8 Aug	31	59	39	18	40	187	37.4
3	11 Aug	57	74	80	67	67	345	69.0
4	16 Aug	69	61	58	56	23	267	53.4
5	18 Aug	67	64	72	45	62	310	62.0
Total		248	314	310	256	240	1,368	54.7

Table 42 Culex quinquefasciatus Larvae Captured in Larvae Traps
During a 24 Hour Period

Trials *	Date Test Ends	Larvae Trapped Per Container					Captured	
		Trap 1	Trap 2	Trap 3	Trap 4	Trap 5	Total	%
1	4 Aug	71	54	62	74	24	285	57.0
2	8 Aug	47	30	71	67	64	279	55.8
3	11 Aug	56	27	52	88	60	283	56.6
4	16 Aug	62	40	84	75	85	346	69.2
5	18 Aug	65	76	65	43	60	309	61.8
Total		301	227	334	347	293	1,502	60.1

* Each trial tested 5 replicates, each involving one trap per each 27 liter water jar and 100 fourth stage larvae per jar.

Three different types of mosquito emergence traps, two types of oviposition container traps, and four modified electric suction traps (without light) have been developed and partially tested. Thus far, the tests on the emergence, oviposition and electric suction traps have proven inconclusive. Development and testing of traps for sampling A. aegypti densities will continue. The larval trap is scheduled to be placed in the residence of the 100 family units in October 1978 and to be used as a routine sampling device thereafter. After reliable sampling techniques are developed, investigation will be initiated to consider the remaining objectives of this study.

16. Virological and Pathological Observations of Dengue Virus Replication in Subcutaneously Innoculated Rhesus Monkeys

OBJECTIVE: To examine the skin and the local lymph nodes of experimentally inoculated monkeys for virus isolation and pathological evidence of dengue virus replication

BACKGROUND: Experimental studies have shown that dengue virus can be recovered from the site of inoculation and from the local draining lymph nodes of Indian Rhesus monkeys (Macaca malatta) (74). Investigations have not been conducted to determine if pathological changes are associated with virus replication. This study was designed to confirm and to extend the virological findings and to examine the local histopathological changes associated with dengue virus replication. In addition, immunofluorescent and electron microscopic studies aimed at identifying the site of dengue virus replication were conducted.

METHODS: The monkeys employed had been used in malaria studies and all had been found to be free of dengue-2 virus neutralizing antibody. Each experimental monkey (F-77, F-78, F-79) was inoculated at nine different sites via the subcutaneous route with dengue-2 virus (BMSO-76, LLC-Mk₂), 0.5 ml per injection site, (Figure 4). The amount of virus in the inoculum was determined by standard plaque assay at 37°C employing LLC-Mk₂ cell cultures. One control monkey (F-80) was inoculated with virus-free LLC-Mk₂ tissue culture fluid. Each monkey was anesthetized with phencyclidin hydrochloride and elliptical biopsy specimens were obtained from the skin at 30 minutes, 24, 48, 72 and 96 hours and at days 11 and 15 post-inoculation. Biopsy specimens of local lymph nodes were obtained at 24, 48, 96 hours and on day 7 post-inoculation. Each biopsy specimen was divided into four parts.

One part of each specimen was placed in 4 ml of Hanks balanced salt (HBS) medium supplemented with 10% calf serum. Specimens were processed for virus isolation according to the explant culture technique (75) of Marchette. Fragments of tissues were washed twice in phosphate buffered saline (PBS) pH 7.9, minced with sterile scissors, and then washed a third time in PBS. The minced tissue was suspended in 4.0 ml of medium 199, 15% calf serum, 1% glutamine and 200 units of penicillin/ml and 200 ug of streptomycin/ml. Each tissue suspension was inoculated onto LLC-Mk₂ cell cultures and incubated at 37°C for 7 days. Medium of cell cultures was removed and replenished every 3 days. Virus isolation attempts were conducted on this medium. On day 7 post-inoculation, cell cultures were subjected to a freeze-thaw cycle and submitted for virus isolation studies. Suspensions of medium and cell cultures were tested for virus by the direct and delayed plaque assay technique.

The remaining portions of each biopsy specimen were sent to the Department of Pathology, Ramathibodi Hospital, Mahidol University for electron microscopy, histopathology and for direct fluorescent antibody studies. One portion was dehydrated at 4°C in a graded series of alcohol and then embedded in epoxy resin. Ultrathin sections were examined with a Hitachi electron microscope, Model HU-12A OY HS-8. Histopathology studies were performed on 10% buffered formalin fixed portions. Specimens for direct F.A. investigation were quick frozen in a dry ice - isopentane mixture. Sections, 4-6 u thick, were made at -20°C with a cryostat, placed on slides, and air dried. Slides were fixed in 2-octanol at -30°C for 30 minutes, dried at 4°C for one hour and stained with Fitc labeled dengue virus antibody.

All monkeys were examined on the day before inoculation and daily throughout the experiment. Blood was obtained from each monkey on day 0 pre-inoculation and on days 1 through 11, and on days 15, 30 and 60 post-inoculation.

Each blood specimens was submitted for hematology studies to determine hemoglobin, hematocrit, white blood count and platelet count. In addition, serum and plasma were tested for antibody, SGOT, SGPT total protein level and for virus, respectively. Virus isolation attempts were performed on peripheral blood leukocytes and on plasma by direct and delayed plaque assay employing LLC-Mk₂ cell cultures at 37°C. Serology was performed by hemagglutination inhibition and complement fixation tests employing Dengue 1-4 and Chikungunya antigens. The plaque reduction neutralization test was performed against dengue-2 virus.

Table 43 Specimens from Monkeys Yielding Dengue-2 Virus

Monkey No.	Inoculation	Day Positive Virus Isolation			
		Plasma	Leukocytes	Skin	Lymph node
F-77	BM50-76 Mk ₂ -2(D-2) 1.1x10 ⁵ PFU SQ x 9	1,4,5,6	-	4,5	4,7
F-78	BM50-76 Mk ₂ -2(D-2) 1.1x10 ⁵ PFU SQ x 9	2,4	5	1,2,7	2,7
F-79	BM50-76 Mk ₂ -2(D-2) 1.1x10 ⁵ PFU SQ x 9	2,4,5	-	3	-
F-80	Uninfected Mk ₂ culture fluid SQ x 9	3,4,5,6	-	5,7	-

Table 44 Reciprocal Hemagglutination Inhibition Titers by Day Following Inoculation of Monkeys with Dengue-2 Virus

Monkey Number	Day Following Inoculation	Reciprocal Hemagglutination Inhibition Titers				
		Den-1	Den-2	Den-3	Den-4	CHIK
F-77	D-0	<10	<10	<10	<10	<10
	D-15	320	1280	1280	2560	<10
	D-30	80	320	160	640	<10
	D-60	40	160	80	320	<10
	D-180	80	320	160	320	<10
F-78	D-0	<10	<10	<10	<10	<10
	D-15	160	640	640	2560	<10
	D-30	80	160	320	640	<10
	D-60	40	160	80	320	<10
	D-180	40	80	80	320	<10
F-79	D-0	<10	<10	<10	<10	<10
	D-15	160	640	320	1280	<10
	D-30	80	320	160	640	<10
	D-60	80	160	80	320	<10
	D-180	80	160	160	320	<10
F-80	D-0	<10	<10	<10	<10	<10
	D-15	80	320	160	640	<10
	D-30	80	160	160	320	<10
	D-60	40	160	40	320	<10
	D-180	20	80	40	40	<10

* Inoculum BM50-76 (LLC-Mk₂-2) 1.1×10^5 PFU

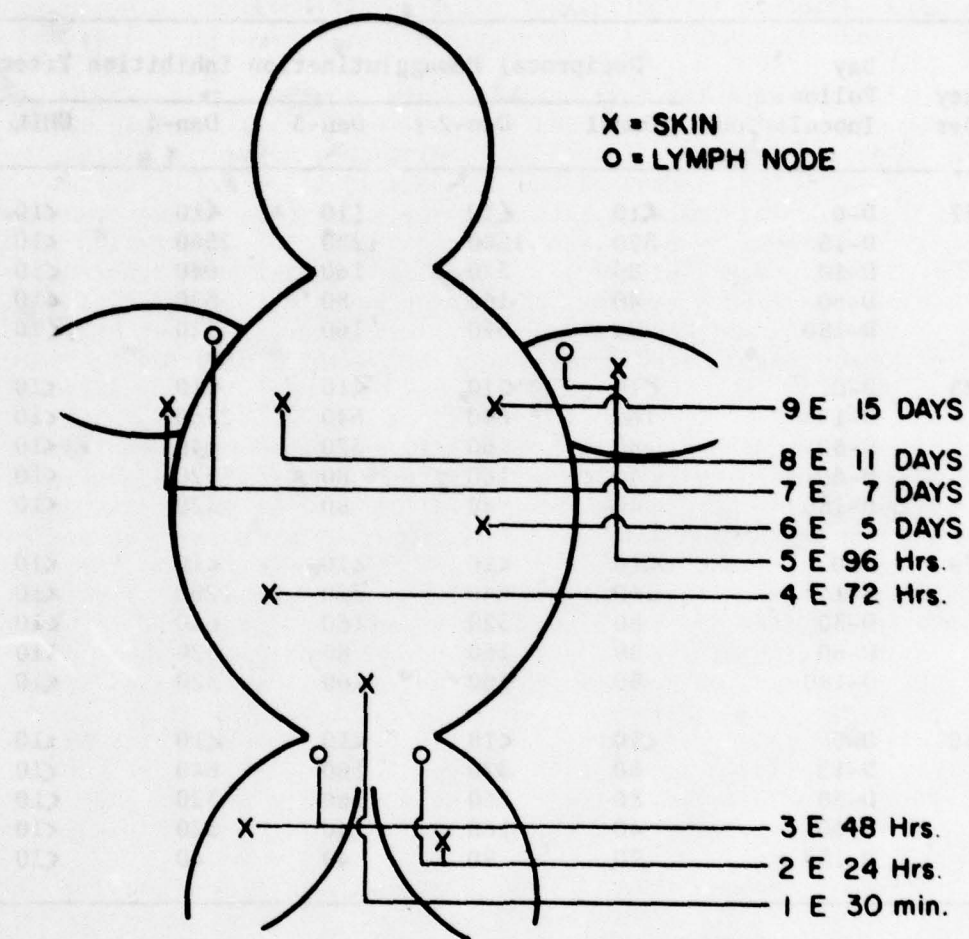


FIGURE 4 SKIN AND LYMPH NODE BIOPSY SITES

RESULTS: Hematologic and Biochemical findings were normal. The results of virus isolation studies performed on monkeys after inoculation of 1.2×10^5 plaque forming units (PFU) of dengue-2 virus per site are presented in Table 43.

Virus was recovered from plasma and skin of all monkeys, whereas leukocytes of only one monkey and lymph nodes of two monkeys yielded virus. The recovery of virus from skin and plasma of the control monkey was probably due to either inadvertent inoculation with a syringe containing virus or contamination of surgical tools used during the performance of biopsies. Lymph nodes taken from this monkey on day 1 and 2, before detectable viremia, served as negative controls for pathology studies. All infected monkeys developed broadly cross-reactive HI antibody to all 4 dengue virus serotypes (Table 44).

Ultra-structural studies of the cells of the primary lymph nodes showed the consistent observation of one or a few viral lattice crystals in the cytoplasm of occasional reticulum cells; in the cytoplasm of the endothelial cells of the capillaries and post-capillary venules, in the cytoplasm of the macrophages, and in the cytoplasm of lymphocytes in the cortical and paracortical areas. Some plasma cells also were observed to contain the crystals. The lattice crystals measured 0.1 to 3 μ m in size, consisted of individual spherical dense particles of 30-35 nm in a paracrystalline arrangement, and were usually enclosed by a membranous structure associated with the cisterna of the rough endoplasmic reticulum. These viral crystals were not seen in control lymph nodes. Results of histopathology and immunofluorescent studies are not yet complete.

17. Evaluation of *Aedes aegypti*, *Armigeres subalbatus*, and *Toxorhynchites splendens* as Bioassay Hosts for Dengue Viruses

OBJECTIVES:

1. To determine the feasibility and effectiveness of employing *Toxorhynchites splendens* as a bioassay host for dengue viruses.
2. To compare *Aedes aegypti* and *Armigeres subalbatus* to *Toxorhynchites splendens* for detection and propagation of dengue viruses.

BACKGROUND: The mosquito species *A. albopictus* has been reported to be a more sensitive bioassay host than conventional cell culture techniques for detecting and propagating dengue viruses (76). Studies conducted at AFRIMS to develop the mosquito assay employing *A. aegypti* have yielded inconsistent results. During 1977, investigation was initiated to assess *T. splendens* as an alternative bioassay host for dengue virus serotypes. The large size of

T. splendens allows for an increase in the volume of inoculum from .17 u/l for individual A. aegypti to .85 u/l for the former species. However, the exceptionally long development period of T. splendens has posed a question regarding the feasibility of producing adequate numbers for employment as a bioassay host for dengue viruses.

METHODS: Male A. aegypti were obtained from colonies established and maintained in the Department of Entomology, AFRIMS. The new colony, 10th to 12th generation, was established from larvae collected in Bangkok during August and September 1977. The old colony, unknown generation, originated from Samui Island, Thailand, June-July 1968. The T. splendens colony, unknown generation, was established from larvae that were collected in Bangkok during July 1976. Male Armigeres subalbatus, unknown generation, were obtained from a colony established during 1966 from specimens provided by the U.S. Army Medical Research Unit, Kuala Lumpur, Malaysia.

Mosquitoes of each species were 3 to 5 days old when employed in experiments. A. aegypti and Ar. subalbatus were reared and maintained according to standard laboratory procedures. A. aegypti larvae were provided continuously to T. splendens larvae and the diet for adults was honey. The procedures for rearing and maintaining T. splendens were modified in an attempt to increase the yield of this species. The oviposition substrate was changed from bamboo cups to photography trays having a black interior. First instar larvae were transferred to individual 9 dm. vials instead of waiting to the third instar stage of development to avoid losses due to cannibalism. Currently, T. splendens are being maintained in 45 x 45 x 45 cm. cages; however, more recent observations suggest that this species will reproduce in smaller cages. The latter is being evaluated in order to increase rearing space.

The origins and passage levels of dengue stock viruses employed are listed in Table 45. Mosquitoes were immobilized and inoculated intrathoracically according to methods described previously (77). Usually five or more T. splendens and 20 to 25 A. aegypti were inoculated with each virus dilution and/or each human leukocyte suspension, 0.85 ul and 0.17 ul per individual mosquito, respectively. Virus dilutions and leukocyte suspensions were prepared in RPMI 1640 medium that was supplemented with heat-inactivated fetal calf serum (FCS), final concentration of 10%, 200 units of penicillin/ml., and 150 ug of streptomycin per ml. After a 14 day incubation period at 32°C, mosquitoes were stored at -70°C for virus assay. The leukocyte suspensions were obtained from the blood of clinically diagnosed DHF patients of the Children's Hospital as described

elsewhere in this Report. All leukocyte suspensions had been assayed for dengue viruses in LLC-Mk₂ cell cultures; however, the technician was not aware of the results at the time specimens were tested for virus by mosquito inoculation.

Human anti-serum that had a hemagglutinating inhibition titer of 1:640 or greater to all 4 dengue virus serotypes was obtained from patients of the Children's Hospital. Anti-serum shown to be negative for hepatitis B surface antigen by radio immune assay was pooled and labeled with fluorescent isothiocyanate (FITC). Tissue imprints of squashed mosquito heads were prepared and assayed for virus by the fluorescent antibody technique as described previously (77). A 1:2 dilution of conjugated antiserum was used throughout the study.

Tissue smears of mosquito heads were examined for fluorescence with the 10 x and 25 x objective of a Leitz fluorescent microscope equipped with a vertical illuminator. The thorax-abdomen portions of mosquitoes for each virus dilution and for each leukocyte suspension were pooled, and disrupted by sonic energy in the presence of 1.5 ml. of RPMI 1640 medium, 10% fetal calf serum, 500 units of penicillin/ml. and 500 ug of streptomycin/ml. Thorax-abdomen suspensions were spun for 30 minutes at 10,000 RPMS in a 4°C centrifuge. Each suspension was tested for virus in LLC-Mk₂ cells by direct plaque assay.

RESULT: Comparative results of the propagation of high and low passage dengue viruses in A. aegypti and T. splendens are presented in Tables 46 and 47. The dilution of low and high passages viruses that yielded fluorescence in each mosquito species was approximately the same, except with high passage dengue 4 virus, which produced fluorescence at a higher dilution in T. splendens than did low passage dengue 4 virus. The titers of dengue viruses in each mosquito species varied slightly. Dengue-4 produced lower titers as well as inconsistent FA results in both A. aegypti and T. splendens. Apparently, the problem was related to the batch of FITC labeled antiserum as virus was recovered in most cases from thorax-abdomen suspensions in the absence of fluorescence. Furthermore, the use of a newly prepared batch of FITC labeled dengue virus antiserum led to a marked increase in fluorescence. The extent and intensity of fluorescence observed in infected mosquitoes appeared to be related to the quantity of virus in each mosquito species. Suspensions prepared from thorax-abdomen of T. splendens consistently yielded more virus than similar A. aegypti suspensions. Fluorescence observed in mosquito head smears was virus specific as indicated by the recovery of virus in LLC-Mk₂ cells from corresponding suspensions prepared from pooled thorax-abdomens. Perinuclear staining was the

Table 45

Dengue Viruses Used to Inoculate Aedes aegypti,
Armigeres subalbatus and Toxorhynchites splendens

Serotype	Date Isolated	Passage Level
Dengue-1 (D75-001)	1975	SMB-03
Dengue-1 (Hawaii)	1944?	SMB-16
Dengue-2 (CH3379)	1974	SMB-05
Dengue-2 (New Guinea)	?	SMB-29
Dengue-3 (77-2797)	1977	SMB-05
Dengue-3 (H87)	1956	SMB-25
Dengue-4 (D77-050)	1977	SMB-03
Dengue-4 (H241)	1956	SMB-32

Table 46

Comparative Titration of High and Low Passage
Dengue Viruses in Aedes aegypti Mosquitoes

Dengue Virus Serotype	Passage	<u>A. aegypti</u>	Undil	Log ₁₀ Dilutions					
				1	2	3	4	5	6
D-1	SMB-16	Old colony	5/6*	6/6	6/6	6/6	4/6	0/6	0/6
D-1	SMB-16	New colony	5/6	6/6	5/6	6/6	5/6	0/6	0/6
D-1	SMB-02	New colony	6/6	6/6	6/6	6/6	3/6	1/6	0/6
D-1	SMB-02	Old colony	6/6	6/6	6/6	6/6	3/6	1/6	0/6
D-2	SMB-29	Old colony	6/6	4/6	6/6	6/6	3/6	0/6	0/6
D-2	SMB-29	New colony	6/6	6/6	6/6	6/6	4/6	0/6	0/6
D-2	SMB-5	New colony	6/6	6/6	6/6	6/6	0/6	0/6	0/6
D-2	SMB-5	Old colony	6/6	6/6	6/6	6/6	1/6	0/6	0/6
D-3	SMB-25	Old colony	4/6	6/6	5/6	5/6	1/6	0/6	0/6
D-3	SMB-25	New colony	6/6	5/6	5/6	5/6	6/6	0/6	0/6
D-3	SMB-5	New colony	ND**	6/6	6/6	6/6	3/6	0/6	0/6
D-3	SMB-5	Old colony	ND	6/6	6/6	6/6	2/6	0/6	0/6
D-4	SMB-32	Old colony	0/6	3/6	0/6	1/6	1/6	0/6	0/6
D-4	SMB-32	New colony	1/6	0/6	0/6	0/6	0/6	0/6	0/6
D-4	SMB-32	Old colony	6/6***	6/6	5/6	3/6	1/6	0/6	ND
D-4	SMB-32	New colony	4/4***	6/6	5/6	2/6	0/6	0/6	0/6
D-4	SMB-03	New colony	6/6***	6/6	6/6	5/6	1/6	0/6	0/6
D-4	SMB-03	Old colony	6/6***	5/6	5/5	1/5	0/5	0/5	0/5

* Number of mosquito head smears showing fluourescence/number of mosquito head smears assayed for virus.

** Not done

*** Results of more recent experiments employ a new batch of FITC labeled dengue virus antiserum.

Table 47

Comparative Titration of High and Low Passage
Dengue Viruses in Toxorhynchites splendens

Dengue Virus Serotype	Passage	Log ₁₀ Dilutions						
		Undil	1	2	3	4	5	6
D-1	SM-16	4/4*	4/4	4/4	4/4	4/4	2/4	ND**
D-1	SM-03	3/3	3/3	4/4	4/4	4/4	2/4	ND
D-2	SM-29	ND	ND	4/4	4/4	4/4	3/4	0/4
D-2	SM-05	ND	ND	4/4	4/4	4/4	2/4	0/4
D-3	SM-25	4/4	4/4	4/4	3/4	3/4	ND	ND
D-3	SM-05	ND	5/5	4/4	5/5	3/4	1/4	0/4
D-4	SM-32	1/4	1/4	1/4	0/4	0/4	0/4	ND
D-4	SM-34	1/4	2/4	1/5	0/4	0/4	0/4	0/4
D-4	SM-32	4/4***	3/4	4/4	3/4	3/4	3/4	ND
D-4	SM-34	3/4***	4/4	4/4	3/4	3/4	3/4	ND

* Number of mosquito head smears showing fluorescence/number of mosquito head smears assayed for virus

** Not done

*** Results of more recent experiments employing a new batch of FITC labeled dengue virus antiserum.

most common type of virus-specific fluorescence observed in mosquito head smears. Occasionally fluorescence was observed as granule-and/or flake-like particles scattered over the surface of tissue smears prepared from infected and uninfected mosquitoes. Virus was not detected in thorax-abdomen suspensions associated with head smears that exhibited the latter type of fluorescence. The head smears prepared from Ar. subalbatus that were inoculated with dengue-1, 3 and 4 viruses failed to fluoresce; however, virus was recovered from corresponding thorax-abdomen suspension of these mosquitoes. Dengue-1 and 4 viruses was recovered from mosquitoes through 10^{-3} dilutions of the inoculum and dengue-3 virus was recovered through the 10^{-4} dilution of the inoculum. Apparently the failure to observe fluorescence was not related to the FITC labeled antiserum as aliquots of the same batch gave specific fluorescence for control smears of all 4 dengue viruses in concurrent experiments involving A. aegypti and T. splendens.

A total of 127 human leukocyte suspensions were tested for dengue virus by intrathoracic inoculation of A. aegypti. Of these suspensions, nine were positive for virus by the F.A. technique. Eight of nine abdomen-thorax suspensions corresponding to the F.A. positive head smears yielded plaques in LLC-Mk cells. In addition, virus was detected in 2 thorax-abdomen suspensions, but no evidence of fluorescence was observed in corresponding head smears. The plaque forming units (PFU) for 2 thorax-abdomen suspensions were too numerous to count, while the count for 8 suspensions ranged from 2 to 89/.3 ml of inoculum. The number of head smears that were F.A. positive for each leukocyte suspensions varied from 1 of 5 to 6 of 6. Of the above 127 leukocyte suspensions, 23 dengue viruses were isolated by direct and delayed plaque assay employing LLC-Mk₂ cells cultures. All viruses detected by intrathoracic inoculation of A. aegypti were also isolated in LLC-Mk₂ cell cultures.

Of the above 127 leukocyte suspensions that were assayed for virus in A. aegypti, 26 have been tested in T. splendens. This included the 23 suspensions that were positive for dengue virus by cell culture assay. Fluorescence was observed in head smears for 18 of the 26 suspensions. Twelve of the 26 corresponding thorax-abdomen suspensions yielded virus by cell culture assay. In addition, 2 thorax-abdomen suspensions were positive for virus even though corresponding head smears were F.A. negative. The plaque count for 10 thorax-abdomen suspensions was too numerous to count and 13 and 30 for the other two suspensions that yielded viruses. Evidence of virus infection was not detected in T. splendens following inoculation of this species with 3 of the 26 suspensions that were negative for virus by cell culture assay.

Although the same leukocyte suspensions were tested for dengue viruses by cell culture assay and by the mosquito inoculation technique, the findings must be interpreted with caution due to the inconsistencies in the testing and treatment of specimens. Leukocyte suspensions were first assayed in LLC-Mk₂ cells which required one or more freeze-thaw cycles. The suspensions were then stored at -70°C for one week to 4 months prior to inoculation of mosquitoes. That this may have altered the infectivity properties was suggested by the failure to reisolate dengue viruses by direct and delayed plaque assay from 9 leukocyte suspensions that were to be employed in another study. The presence of virus specific-like fluorescence in mosquito head smears prepared from dengue virus inoculated mosquitoes and the absence of detectable virus in corresponding thorax-abdomen suspensions has been reported previously (77). However, the frequency was exceptionally low compared to that in data obtained for dengue viruses and T. splendens. Such findings were not observed in T. splendens that were inoculated with low passage mouse brain propagated dengue seed viruses. Nor was perinuclear fluorescence observed in control head smears prepared from T. splendens. Further investigations will be required to assess T. splendens as a bioassay host for dengue viruses. A. aegypti will no longer be considered for dengue virus assay since an adequate number of T. splendens are now available. Modification of rearing technique has increased the yield of this species from an average of 60 to 200 per week.

18. Relationship of Temperature to the Replication of Dengue Viruses in LLC-Mk₂ Cell Cultures

OBJECTIVES: To determine the plating efficiency, plaque morphology, and kinetics of replication of dengue viruses at different temperatures.

BACKGROUND: Each of the currently recognized serotypes of dengue viruses has been associated with disease of man that differs widely in severity (78, 56). One hypothesis that has been advanced to explain the variation in severity of disease is that dengue viruses differ in regard to virulence properties (79). A corollary to this hypothesis stated that the determinant of severe dengue syndrome was related to the surface antigens of dengue viruses. Although the antigenic and biological properties of these viruses differ, such markers have not proven to be associated with a particular human disease syndrome (80, 81).

Table 48. Replication of dengue-3 viruses in LLC-Mk₂ cells at different temperatures.*

Dengue-3 Strain	Temperature(°C)	Plaque forming units			
		Undiluted	1	2	3
2878 (Nonfatal case)	32	TNTC**	TNTC	TNTC	78
		TNTC	TNTC	TNTC	81
	35	TNTC	TNTC	105	16
		TNTC	TNTC	80	20
2797 (Fatal case)	32	TNTC	66	07	00
		TNTC	86	09	00
	35	55	10	00	00
		-	6	00	00

* Plaques were not observed in LLC-Mk₂ cells at 39°C.

** To numerous to count.

Investigations have shown that the replication of dengue viruses as well as strains of each serotype appeared to be influenced by temperature (80). In addition, more recent studies have revealed that dengue viruses, especially dengue-2, were comprised of subpopulations that differed considerably in regard to in-vitro replication at different temperatures (82). Virulence for mice was found to vary according to the temperature that was permissive for the replication of subpopulations of this virus. This study was initiated to determine if temperature requirements for in vitro replication of dengue viruses could be used as a marker to differentiate these viruses in regard to their pathogenicity for man.

METHODS: Wild dengue viruses were obtained in conjunction with other studies from clinically diagnosed dengue fever and dengue hemorrhagic fever patients admitted to the children's Hospital, Bangkok. Two dengue-3 viruses were isolated from patients admitted to the Khon Kaen Hospital, Khon Kaen. If possible, strains of each dengue virus serotype were obtained, including strains from patients who had grade I illness and strains from patients who had grade IV and/or from fatal cases. The clinical history and the identity of each dengue virus was obtained from clinical records maintained in the Department of Virology, AFRIMS. The plating efficiency of each virus was determined by assay of original and/or log₁₀ dilutions of leukocyte specimens by the direct plaque technique at 32, 35 and 39°C in LLC-Mk₂ cell cultures. The number of plaque forming units (PFU) and the diameter of plaques were recorded for viruses at each temperature.

Virus replication curves were established for selected low passage suckling mouse brain propagated dengue viruses at 32°C, 35°C and 37°C. Tube cultures of LLC-Mk₂ cells were inoculated with a concentration of virus to yield a multiplicity of infection of 0.1. at 12 hour intervals, glass beads were added to two or more cultures for each temperature. Cell cultures were then disrupted by vigorous agitation employing a vortex mixer. After adding 0.5 ml of fetal calf serum (heat treated at 56°C x 30 min) to each suspension, the contents of each tube were pooled and 0.5 ml aliquots were stored at -70°C. Suspensions were assayed for virus in LLC-Mk₂ cells by direct plaque assay. Plaque forming units were recorded on day 6 post inoculation.

RESULTS: Eleven original human plasma and/or leukocyte suspensions from which dengue viruses had been isolated during 1977 were selected for plating efficiency studies. Included were strains of dengue 2, 3 and 4 viruses that were associated with different grades

Table 49. Replication of virus isolates at 37°C in LLC-Mk₂ cells.

Plasma Number	Number of passages							
	P-0	P-1	P-2	P-3	P-4	P-5	P-6	P-7
2737/77	108*	02	3,6	0				
2713/77	TNTC**	80x10 ³	9x10 ²	0				
2817/77	06	1x10 ²	3x10 ³	2x10 ²	2x10 ¹	0		
2051/75***						6x10 ²	27x10 ¹	0
H58525/77	4	1x10 ¹	1x10 ¹	0				

* Plaque forming units obtained by either direct or delayed plaque assay from original specimen.

** Too numerous to count.

*** Data prior to passage 5 was not available.

Table 50. Replication of virus isolates at 32°C in LLC-Mk₂ cells.

Plasma Number	Number of passages							
	P-0	P-1	P-2	P-3	P-4	P-5	P-6	P-7
2737/77	TNTC	46x10 ³						
2713/77	TNTC	11x10 ¹	87x10 ²	50x10 ³				
2817/77	-*			84x10 ¹	9x10 ³	2x10 ³		
2051/75**	-*						7x10 ¹	5x10 ²
H58525/77	-*			83x10 ¹	4x10 ³	1x10 ³	1x10 ³	132x10 ³

* Original of these isolates were not available.

** Isolate number 2051 yielded 8x10², 11x10³, 42x10³ at LLC-Mk₂ passage 8, 9 and 10, respectively.

of human illness. Dengue-1 virus was not available. The plating efficiency of 9 strains including the five dengue-2 and the four dengue 4 strains could not be determined due to unsuccessful attempts to reisolate virus from original leukocyte suspensions employing LLC-Mk₂ cells. Dengue-3 virus was reisolated from each of two original human plasma specimens. As shown in Table 48, the PFU yield for both strains was greater at 32°C. Although the titer was slightly higher for strain #2378 the difference in plaque yield for each strain at 32°C and 35°C was comparable. The diameter of PFU for each strain at 32°C and 35°C ranged from 1 to 2 mm.

The observation that the optimal temperature for replication of the dengue-3 strains was 32°C proved to be helpful for routine isolation and identification of dengue viruses. As shown in Table 49, passage of five virus isolates at 37°C in LLC-Mk₂ cells to increase titers for virus identification resulted in a complete loss of infectivity. Subsequently, the same 5 isolates were assayed in LLC-Mk₂ cells at 32°C employing the original specimens and/or different LLC-Mk₂ passage material as a source of virus. The results presented in Table 50 showed each virus isolate to attain peak titer after one or two passage in at 32°C. In contrast to results obtained at 37°C, the titers of each virus isolate at 32°C remained approximately the same regardless of the passage level. Virus isolate #2737 was of particular interest in that the number of PFUs increased from 108 per 0.3 ml at 37°C too to numerous to count (TNTC) at 32°C where as the plaque count remained TNTC for #2713. All five isolates were subsequently determined to be dengue 3 virus. The clinical histories of the patients from whom the viruses were isolated were not available.

The rate and magnitude of replication of 2 strains of dengue-4, and one each of dengue-1 and dengue-2 that originated from patients, grade I and grade II illness, were determined at 32°C, 35°C and 37°C. No apparent difference was noted for the replication of each virus at the different temperatures, except for dengue 2 virus that was detected 12 to 24 hours prior to the recovery of the other viruses and peak titers for dengue-1 was attained 36 to 48 hours later than dengue 2 and dengue 4 viruses.

This investigation will be continued to further define the relationship of temperature to the replication of selected strains of dengue viruses.

19. A longitudinal Serological Study of A Lower Socioeconomic School Population

OBJECTIVES:

1. To describe the prevalence of serologic evidence of previous arboviral and hepatitis B viral infections in a susceptible school population.
2. To determine the incidence of clinical and subclinical infections with the above agents during the "disease season" and during the period of the year without a large amount of clinical illness.
3. To investigate socioeconomic parameters possibly related to infection occurrence.

BACKGROUND: Arboviral Infections: A pre-and post-dengue transmission season study of 1131 Bangkok children, ages 1-14 years, and residing in 19 different study areas, was carried out in 1962 (57). During the one-year study period, five children (0.4%) had serologic evidence of recent Dengue Type 1 infection (HI titer $\geq 1:640$) and had been hospitalized with hemorrhagic fever. Seventy children (6.2%) had such serologic evidence without overt hemorrhagic fever. The percentage of children with Dengue Type 1 antibody titers of $\geq 1:640$ ranged from 3% to 9% at the various ages. Over 40% of all children had detectable HI antibody to this virus, the percentage rising with increasing age and decreasing socioeconomic status.

Six hundred and twenty-eight children with a diagnosis of hemorrhagic fever were admitted to a clinical, virologic, and serologic study carried out from 1962 to 1965 (83). Over 80% of these children had dengue infections proven by virus isolation or serology; a secondary type antibody response occurred in 85% of these. The data suggested the hypothesis that the relative risk of dengue hemorrhagic fever and dengue shock syndrome is higher in children with secondary dengue infections. To date, dengue hemorrhagic fever remains the greatest cause of hospitalization and death among children in Thailand under the age of 15 years.

Hepatitis B Viral Infections: Study of a well-defined population of lower socioeconomic Bangkok residents (Huay Khwang) (84) has shown that 8.2% of the population have Hepatitis B surface antigen (HB_sAg) in their blood. Children in the 1-4 year age group have a 4.5% rate of antigen positivity. There was no significant

difference in antigen prevalence between age groups. Antibody prevalence, on the other hand, was noted to increase rapidly from 15.4% to 48.9% between the ages of one and 19 years. These data suggested that the risk of acquiring hepatitis B antigen, but not antibody, was associated with the family unit.

METHODS: Students attending the Phibunprachasan School in the Din Daeng area of Bangkok were studied. A blood sample was collected in June 1977 prior to the "dengue season" (i.e. June-October) from 1988 students. One thousand nine hundred and three of these students had a repeat sample drawn after the "dengue season" in January 1978. A third blood sample was collected from 1249 students in June 1978. Basic demographic data on family size, family income, age and sex of family members, and the rank of the index child in the family were collected along with a medical history of the school child. Children who were absent from school for three or more days were visited in their homes if necessary, by a public health nurse throughout the school year and acute and 14 day convalescent blood specimens were collected on children ill with symptoms possibly referable to a dengue or hepatitis viral infection. If the child was hospitalized, the treating physician was interviewed, acute and convalescent blood samples were collected, and a copy of the child's hospital record was obtained.

All blood samples obtained either were or will be tested for dengue, JEV and Group A arboviral antibodies by hemagglutination-inhibition. Chikungunya virus antigen was used to test for Group A antibodies. Virus isolation was attempted on all specimens obtained during the acute phase of a febrile disease. Sera obtained were also tested for the presence of HB_sAg and anti-HB_s by radioimmune assay. Those with HB_sAg were subtyped and examined for the presence of HB_eAg and anti-HB_e. Serum specimens were collected from the families of index children who were HB_sAg positive and were tested in the same manner as that of the school child.

RESULTS:

Arboviral Infections: Table 51 presents the prevalence of positive titers (≥ 1:20) by age and sex of the school children. Medical history of the child, family income, and number of children in the family did not appear to have a significant association with the distribution of antibody to either Group A or Group B arboviruses.

Table 51 Prevalence of Antibody to Group A and B Arboviruses
Phibunprachasan School, Bangkok, Thailand June 1977

GROUP A

Age	No. of males in the study	No. of pos. males	No. of females in the study	No. of pos. females	Total chil- dren in study	Total pos. chil- dren	% pos. chil- dren
4	14	-	25	-	39	-	-
5	31	4	37	3	68	7	10
6	57	-	31	3	88	3	3
7	75	5	113	10	188	15	8
8	115	11	90	4	205	15	7
9	116	9	113	10	229	19	8
10	123	16	126	22	249	38	15
11	110	19	110	19	220	38	17
12	120	22	128	29	248	51	21
13	105	27	157	44	262	71	27
14	45	13	82	22	127	35	28
15	17	7	28	3	45	10	22
16	2	-	6	4	8	4	50
17	-	-	2	-	2	-	-

GROUP B

4	14	4	25	3	39	7	18
5	31	15	37	14	68	29	43
6	57	23	36	15	93	38	41
7	75	32	113	50	188	82	44
8	115	63	90	40	205	103	50
9	116	66	113	62	229	128	56
10	123	74	126	73	249	147	59
11	110	57	110	60	220	117	53
12	120	76	128	76	248	152	61
13	105	76	157	115	262	191	73
14	45	34	82	53	127	87	69
15	17	15	28	17	45	32	71
16	2	2	6	5	8	7	88
17	-	-	2	2	2	2	100

Table 52 Prevalence of Dengue Type 2 Antibody
by Family Rank Order

Rank of child	Antibody Titer	
	$\leq 1:20$	$\leq 1:20$
1	205	173
2+	956	611

df = 1

$\chi^2 = 5.53$

y p < .025

Relative risk = 1.08

The group of children with one or more positive titers averaged approximately 16 months older than those without, but male/female differences were not found. Children, other than the eldest or only child, all have approximately the same risk of antibody acquisition. However, the eldest child's experience is moderately different from children with a lower rank in their family. (By "rank" is meant the order of the child in the family (e.g. the eldest child is ranked 1 and the fifth child in the family is ranked 5)). The eldest child appears to have a slightly increased risk of antibody acquisition over children with older siblings. Table 52 presents the data on the prevalence of Dengue Type 2. This finding is consistent in greater or lesser degree in all the Group B arboviruses tested.

Approximately 16% of the children at Phibunprachasan School experienced an infection with either a Group A or Group B arboviral agent during the 1977 "dengue season". Of 824 students who were seronegative for any Group B agent, 110 (13.3%) sustained a primary type titer rise (maximum: 1:640), and an additional nine (1.1%) sustained a secondary type titer rise (minimum: 1:1280). Of the 1065 students with at least a 1:20 titer to one or more of the Group B arboviruses prior to the "dengue season", 175 (16.4%) sustained at least one fourfold or greater titer rise between the June 1977 and January 1978 blood specimens. The distribution of these infections between the various viruses is presented in Tables 53 and 54. The corresponding values for Group A arboviral agents was 22 primary and two secondary responses out of 1540 susceptibles (1.6%) and 10 infections out of the 349 students with pre-existing antibody (2.9%).

Neither family income, geographic location of the family, number of children in the family, sex of the child nor the rank of the child within the family appeared to play a significant role in the distribution of infections.

The age distribution of cases of infection, and to a lesser extent the sex distribution, was a reflection of the Prathom or school class in which the child was enrolled. For example, Table 55 presents the data on Group A infections. Since Prathom 6 is predominately aged 11-13, this focus of infection is represented in the age distribution. Similar mini-epidemics can be documented for Dengue Types 1, 3 and 4.

As can be seen from Table 54 the majority of secondary infections demonstrate fourfold or greater titer rises to multiple closely related Group B arboviruses. A minority however can be labeled

Table 53. Primary Cases of Group A and Group B Arbovirus Infections in Children of Phibunprachasan School, Bangkok, Thailand, Jun 77 - Jan 78

	Number of Infections
Group A	22
Group B	110
Dengue 1	6
Dengue 2	10
Dengue 3	6
Dengue 4	61
JEV	1
Indistinguishable Combinations of 2 Group B	
Dengue 1,2	1
Dengue 1,3	1
Dengue 2,3	2
Dengue 2,4	1
Dengue 3,4	11
Dengue 4, JEV	6
Indistinguishable Combinations of 3 Group B	
Dengue 1,3,4	1
Dengue 2,4,JEV	1
Dengue 3,4,JEV	2
	132

Table 54 Secondary Cases of Group A and Group B Arbovirus Infections in Children of Phibunprachasan School, Bangkok, Thailand, June 77 - Jan 78

Virus	Number of infections by the case definition of 2°	
	Pre-existing antibody	Size of titer rise alone (1:1280 as minimum)
Group A	10	2
Group B	175	9
Dengue 1	4	-
Dengue 2	9	-
Dengue 3	14	-
Dengue 4	20	4
JEV	2	-
Indistinguishable Combinations of 2 Group B		
Dengue 1,2	2	-
Dengue 1,3	1	1
Dengue 1,4	1	-
Dengue 2,3	1	-
Dengue 3,4	4	-
Dengue 4, JEV	-	2
Indistinguishable Combinations of 3 or more Group B	117	2
	185	11

Table 55. Comparison of Prathom 6 and the Remainder of Phibunprachasan School with Regard to Infection with Group A Arbovirus

		Prathom	
		6	Not 6
Infection with Group A Arbovirus	+	13	21
	-	211	1658

$df = 1$
 $\chi^2 = 20.82$
 y $p < .0005$

as to infecting agent based on a single specific titer rise. The sum of primary and secondary infections with an isolated titer rise provide the basis for an age and sex distribution pattern for infections with the individual viral agents. Table 56 presents an example of this pattern method. The effect of isolated outbreaks of clinical and subclinical infection in Prathoms 5 (10-12 year old children) and 1 (7 and 8 year old children) can be seen. The total numbers of cases in these outbreaks are larger than depicted by tables such as Table 56 as most of the secondary infections will be among the indistinguishable combination groups.

Sixteen children were clinically diagnosed as having hemorrhagic fever during the course of the school year. Ten of these showed no serologic evidence of arboviral infection either between acute and convalescent blood specimens or between the surveys. Of the 328 seroconversions (including 23 students who seroconverted to both Groups A and B arbovirus), which occurred during the school year, only 6 (1.8%) were accompanied by clinical evidence of hemorrhagic fever. One of these was Group A disease for a clinical: subclinical ratio of 1:33 and the remainder were Group B disease for a clinical: subclinical ratio of 1:58. Three (60%) of these cases were secondary compared to 62% of all Group B seroconversions being of a secondary type.

Hepatitis B Viral Infections: Evidence of Hepatitis B Virus (HBV) infection was detected in sera of 38.9% of males and 33.3% of females in the population of the Phibunprachasan School sampled during June 1977 (Tables 57 and 58). Considerable variation was observed in the age specific prevalence rates of HBV infection with a gradual increase with age noted in both sexes.

The HBV antibody acquisition rates subsequent to a previous hepatitis B antigenemia for the entire population was 10.3% (Table 59). The difference between males and females was not significant. Antibody acquisition was observed in the age range 7 to 15 for males and 5 to 15 for females. The age-sex incidence of HBV infection, based on the acquisition of either HB_sAg or anti-HB_s is presented in Table 60. Overall, 4.3% of the population acquired HBV infection (either HB_sAg or anti-HB_s) during the period of seven months (June 1977 through January 1978). The acquisition of HB_sAg was 1.87% in males compared to a lower rate in females, 0.56%. No apparent difference was noted between sexes in the acquisition of antibody to HBV infection.

Table 56. Age and Sex Distribution of Primary and Secondary Infections with Dengue Type 4 Arbovirus Phibunprachasan School, Bangkok, Thailand, Jun 77 - Jan 78

Age	Primary Infection		Secondary Infection		Total	
	M	F	M	F	M	F
4	-	-	-	-	-	-
5	1	2	-	-	1	2
6	-	1	-	-	-	1
7	3	9	1	1	4	10
8	4	5	1	-	5	5
9	4	3	1	-	5	3
10	7	4	4	8	11	12
11	-	3	1	4	1	7
12	3	3	1	1	4	4
13	4	4	1	-	5	4
14	-	1	-	-	-	1
15	-	-	-	-	-	-
16	-	-	-	-	-	-
	26	35	10	14	36	49

Table 57. The Prevalence of Hepatitis B Virus Surface Antigen in Students of the Phibunprachasan School, Bangkok, Thailand, June 1977

Age	MALE			FEMALE			TOTAL		
	No. tested	HBsAg *		No. tested	HBsAg *		No. Tested	HBsAg *	
		No.	%		No.	%		No.	%
4	15	2	13.3	31	4	12.9	46	6	13.0
5	30	4	13.3	33	3	9.0	63	7	11.1
6	52	4	7.7	35	1	2.8	87	5	5.7
7	76	9	11.8	102	6	5.9	178	15	8.4
8	113	9	8.0	88	7	7.9	201	16	7.9
9	116	13	11.2	110	7	6.4	226	20	8.8
10	123	15	12.2	127	19	15.0	250	34	13.6
11	104	10	9.6	105	5	4.8	209	15	7.2
12	115	4	3.5	131	7	5.3	246	11	4.5
13	106	10	9.4	151	7	4.6	257	17	6.6
14	42	4	9.5	78	7	9.0	120	11	9.2
15	20	2	10.0	28	6	21.4	48	8	16.7
Total	912	86	9.4	1019	79	7.75	1931	165	8.5

* Based on the Radioimmune Assay (RIA) and Immuno-electrophoresis (IEOP) Tests.

Table 58. The Prevalence of Hepatitis B Surface Antibody (anti-HBs) Students of the Phibunprachasan School, Bangkok, Thailand, June 1977

Age	MALE			FEMALE			TOTAL		
	No. tested	Anti-HBs *		No. tested	Anti-HBs *		No. tested	Anti-HBs *	
		No.	%		No.	%		No.	%
4	15	3	20.0	31	2	6.5	46	5	10.9
5	30	3	10.0	33	3	9.1	63	6	9.5
6	52	5	9.6	35	7	20.0	87	12	13.8
7	76	13	17.3	102	29	28.4	178	42	23.6
8	113	28	24.8	88	13	14.8	201	41	20.4
9	116	30	25.9	110	25	22.7	226	55	24.3
10	123	38	30.9	127	24	18.9	250	62	24.8
11	104	32	30.8	105	24	22.9	209	56	26.8
12	115	43	37.4	131	31	23.7	246	74	30.1
13	106	49	46.2	151	55	36.4	257	104	40.5
14	42	15	35.7	78	28	35.9	120	43	35.8
15	20	10	50.0	28	16	57.1	48	26	54.2
Total	912	269	29.5	1019	257	25.2	1931	526	27.2

* Based on the RIA and IEOP tests.

Table 59. Evidence of HBV Antibody Acquisition*
of HBV Infection During Study Period.
Phibunprachasan School, June 1977-Jan 1978

Age	Male	Female	Total
4	0	0	0
5	0	1	1
6	0	0	0
7	1	1	2
8	1	1	2
9	2	2	4
10	1	0	1
11	1	0	1
12	1	0	1
13	0	1	1
14	0	1	1
15	1	2	3
Total	8	9	17
Percentage of antibody acquisition	9.3	11.4	10.3

* Detection of HB_sAg in 1st bleeding and anti-HB_s
in 2nd bleeding.

Table 60. The Incidence of HBV Infection During Study Period,
Phibumprachasan School, June 1977 - Jan 1978

Age	MALE			FEMALE			TOTAL		
	HB _s Ag	Anti-HB _s	Total	HB _s Ag	Anti-HB _s	Total	HB _s Ag	Anti-HB _s	Total
4	0	1	1	0	1	1	0	2	2
5	1	1	2	0	1	1	1	2	3
6	1	1	2	0	1	1	1	2	3
7	3	0	3	0	3	3	3	3	6
8	0	4	4	1	2	3	1	6	7
9	1	1	2	0	2	2	1	3	4
10	0	2	2	1	3	4	1	5	6
11	1	2	3	0	2	2	1	4	5
12	3	2	5	0	6	6	3	8	11
13	0	1	1	2	2	4	2	3	5
14	1	0	1	0	2	2	1	2	3
15	0	0	0	0	1	1	0	1	1
Total	11	15	26	4	26	30	15	41	56
Rate	11/588	15/588	26/588	4/710	26/710	30/710	15/1298	41/1298	56/1298
Percent	1.87	2.55	4.42	0.56	3.66	4.23	1.16	3.16	4.31

Table 61. Rate of Disappearance of HBV Surface Antigenemia and of HBV Antibody in Students of Phibumprachasan School During Study Period June 1977 - January 1978

Age	MALE		FEMALE		TOTAL	
	HB _s Ag	Anti-HB _s	HB _s Ag	Anti-HB _s	HB _s Ag	Anti-HB _s
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
4	0 (-)	1 (33)	3 (75)	0 (-)	3 (50)	1 (20)
5	1 (25)	0 (-)	0 (-)	0 (-)	1 (14)	0 (-)
6	1 (25)	1 (20)	0 (-)	2 (29)	1 (20)	3 (25)
7	1 (11)	2 (15)	1 (17)	3 (10)	2 (13)	5 (12)
8	0 (-)	2 (7)	0 (-)	1 (8)	0 (-)	3 (7)
9	2 (15)	1 (3)	1 (14)	1 (4)	3 (15)	2 (4)
10	5 (33)	2 (5)	4 (21)	0 (-)	9 (26)	2 (3)
11	3 (30)	0 (-)	2 (40)	3 (13)	5 (33)	3 (5)
12	1 (25)	1 (2)	2 (29)	3 (10)	3 (27)	4 (5)
13	0 (-)	3 (6)	1 (14)	1 (2)	1 (6)	4 (4)
14	1 (25)	1 (7)	2 (29)	4 (14)	3 (27)	5 (12)
15	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Total	15	14	16	18	31	32
Rate(%)	17.4	5.2	20.3	7.0	18.8	6.1

Table 62. Household Contacts of Hepatitis B
Antigenemic Students; Relation of
HB_eAg, Phibunprachasan School

HB _e Ag	No. of students	No. of families* with presence of HBV marker	
		No.	%
Students with HB _e Ag	26	21	80.76
Students without HB _e Ag	13	9	69.23

* Detection of HBV markers in one or more
family members.

p = 0.22 (Fisher's exact test)

Among the study population of this lower socioeconomic group, 17.4% of males and 20.2% of females who were antigenemic in June 1977 spontaneously lost their antigen positivity by January 1978, while 5.5% of males and 7.3% of females lost their antibody during the study period (Table 61). The decline in detectable HB_sAg and anti-HB_s was greater in females than in males; however this difference was not statistically significant.

Study of the hepatitis B e antigen (HB_eAg), in students with HB_s antigenemia, demonstrated an apparent trend for students antigenemic for both HB_sAg and HB_eAg to have a greater chance of having household contacts with evidence of previous Hepatitis B infection (Table 62), but the number of families was too small for demonstration of statistical significance. Further work on this point will be necessary.

Determination of the arboviral and hepatitis B virus antibody status of the third blood sample collected in June 1978 is pending.

20. Seroepidemiologic Survey of Hepatitis B Virus Infection in a Rural Thai Village

OBJECTIVES:

1. To determine the prevalence of hepatitis B surface antigen (HB_sAg) and antibody to hepatitis B surface antigen (anti-HB_s) in a well-defined rural Thai population.
2. To determine if there is a familial clustering of either HB_sAg or anti-HB_s.

BACKGROUND: A study on a well defined urban Thai population (84) has shown an average prevalence of HB_sAg of 8.2% (determined by radioimmune assay) and anti-HB_s of 46.1% (determined by passive hemagglutination). In this population of 697 people age one year to 75 years, the prevalence of HB_sAg was similar throughout all age groups. For anti-HB_s, on the other hand, the prevalence rose from 15.4% in the 1-4 age group to a plateau level of 50 to 65% after the age of 20. A stable rural Thai population was sought to determine the prevalence of HB_sAg and anti-HB_s for comparison with that found in the Bangkok population.

METHODS: The village of Tablan was selected for study. This village was located in Prachinburi province in the Bhu Phram valley. Its population had been included in malaria drug prophylaxis studies for two years. Sera from a portion of the population had been collected for malaria studies at approximately yearly intervals in 1974 and 1975.

Table 63 Prevalence of HB_sAg in Residents of Ban Tablan

Age (Year)	MALE			FEMALE			TOTAL		
	No. Tested	Positive		No. Tested	Positive		No. Tested	Positive	
		No.	%		No.	%		No.	%
0-4	33	0	0	39	1	2.56	72	1	1.39
5-9	59	7	11.86	69	4	5.79	128	11	8.59
10-14	64	3	4.68	66	6	9.09	130	9	6.92
15-19	39	5	12.82	34	3	8.82	73	8	10.96
20-29	56	9	16.07	67	3	4.48	123	12	9.76
30-39	40	5	12.50	45	0	0	85	5	5.88
40-49	36	3	8.33	28	0	0	64	3	4.69
50-59	21	2	9.52	26	2	7.69	47	4	8.51
60+	11	2	18.18	12	0	0	23	2	8.69
Total	359	36	10.03	386	19	4.92	745	55	7.38
Age adjusted prevalence		10.07				4.92			

Table 64. Prevalence of Anti-HB_s in Residents of Ban Tablan

Age (Year)	MALE			FEMALE			TOTAL		
	No. Tested	Positive		No. Tested	Positive		No. Tested	Positive	
		No.	%		No.	%		No.	%
0-4	33	2	6.06	39	7	17.94	72	9	12.50
5-9	59	12	20.33	69	13	18.84	128	25	19.53
10-14	64	24	37.50	66	23	34.84	130	47	36.15
15-19	38	21	55.26	34	9	26.47	72	30	41.67
20-29	56	27	48.21	67	26	38.80	123	53	43.09
30-39	40	20	50.00	45	22	48.89	85	42	49.41
40-49	36	24	66.67	28	12	42.86	64	36	56.25
50-59	21	13	61.90	26	14	53.85	47	27	57.45
60+	11	8	72.73	12	10	83.33	23	18	78.26
Total	358	151	42.18	386	136	35.23	744	287	38.58
Age adjusted prevalence			41.56				35.29		

Table 65 Anti-HB_s Prevalence in Children (under 18 years of age) by the Presence of a HB_sAg Carrier in the Household.

No. of HB _s Ag positive individuals in the family	No. of children	Anti-HB _s positive	
		No.	%
0	284	74	26.1
1 or more	76	31	40.8

df = 1
 $\chi^2 = 5.61$
 y p < .0125

Table 66 HBsAg Prevalence by Family Size

Family size	No. of families	% of families with at least one HBsAg positive individual	No. of people in families of this size	% of individuals HBsAg positive	Mean No. of HBsAg positive individuals in an HBsAg positive family
2-3	54	24.1	132	11.4	1.2
4-5	56	19.6	254	5.5	1.3
6-7	38	28.9	245	6.1	1.4
8-9	7	14.3	59	1.7	1.0
10+	4	25.0	41	4.9	2.0
	159	23.3	731	6.4	1.3

Table 67 Serologic Status HBV Ban Tablan, Thailand

Status 1975	Total	Status 1976	Total
Ag-Ab-	64	Ag-Ab-	52
		Ag-Ab+	10
		Ag+Ab-	2
		Ag+Ab+	0
Ag-Ab+	74	Ag-Ab-	3
		Ag-Ab+	70
		Ag+Ab-	0
		Ag+Ab+	1
Ag+Ab-	15	Ag-Ab-	0
		Ag-Ab+	0
		Ag+Ab-	15
		Ag+Ab+	0
Ag+Ab+	2	Ag-Ab-	0
		Ag-Ab+	0
		Ag+Ab-	1
		Ag+Ab+	1

A census of the village conducted in early 1976 showed the total population to consist of 1,014 people, 503 males and 511 females. Sera were assayed for HB_sAg and anti-HB_s serology by radioimmune assay (AUSRIA II and AUSAB supplied by Abbott Laboratories, North Chicago, Ill.).

RESULTS: Prevalence Data: The age- and sex-specific prevalence rates of HB_sAg for 1976 as detected by RIA are presented in Table 63. While the prevalence rate in males is relatively constant after infancy, the prevalence of HB_sAg is significantly lower in females mainly because of the near total lack of antigenemia in those women over 30, and secondarily because of generally lower prevalence rates in the younger age groups. Although the prevalence rates of HB_sAg for three of the four groups of females from age 5-29 are lower than those of the corresponding males groups, none of these individual differences were sufficient to be termed significant. Age adjustment does not change the prevalence rates to a large degree.

A completely different distribution has been noted for the prevalence of anti-HB_s determined on the same sera. Table 64 presents the age and sex-specific prevalence rates for anti-HB_s. The prevalence rates rose progressively with age in both sexes. Except for the very young and the very old, male rates again were uniformly higher than their female contemporaries, but no significant difference was evident in any individual groups nor in the overall prevalence rate. Age adjustment did not influence the prevalence rates of anti-HB_s in males or females.

Family Interactions: Antibody prevalence tended to cluster in those families with one or more HB_sAg carriers in the family. Table 65 points out the association in children under the age of 18. On the other hand, there was no evidence of the clustering of HB_sAg positive individuals in the family group. As can be seen in the final column of Table 66, the average number of antigenemic individuals changed very little with variation in family size.

In an effort to identify the status of intra-familial transmission, families in which a single parent was HB_sAg positive were sought. Fifteen such families, in which the HB_sAg status of both spouses was known, were found. In all 15 families, it was the father who was antigen positive. This was not surprising, considering the relative lack of female HB_sAg carriers of maternal age as demonstrated in Table 63. In those 15 families, three of 41 children (7.3%) were HB_sAg positive. In 100 families, in which both spouses were HB_sAg negative, 13 of 244 children (5.3%) were HB_sAg positive. There were no families in which both spouses were HB_sAg positive.

There was no evidence that intra-familial transmission was taking place from child to either parent. In families in which one or more children were HB_sAg positive, two of 12 (16.7%) fathers were also positive. In families in which all children tested were HB_sAg negative 14 of 96 (14.6%) fathers were HB_sAg positive. The corresponding figures for mothers were zero out of 20 (0%) and three out of 107 (2.8%) respectively.

Incidence Data: Table 67 presents the relationship of time on the various combinations of HB_sAg and anti-HB_s extant in Ban Tablan in 1975. The time period covered averaged 19 months between blood samples and those 1975 samples which could not be repeated in concert with the corresponding 1976 specimen were eliminated from analysis.

During this period, ten of 79 (12.7%) anti-HB_s negative individuals gained antibody and four of 76 (5.3%) lost a positive antibody titer. Three of 138 (2.2%) HB_sAg negative individuals acquired antigenemia and none of 17 antigenemic individuals became seronegative.

21. Transmission of Hepatitis B Virus by Exposure to Human Semen Containing Hepatitis B Surface Antigen

OBJECTIVE: To test the hypothesis that human semen can transmit Hepatitis B virus (HBV) infection.

BACKGROUND: Epidemiological evidence suggests that HBV may be transmitted by a sexual route. Hepatitis B antigen (HB_sAg) has been detected in semen (85). The present study attempted to determine if human semen can transmit HBV by parenteral and vaginal administration using the gibbon as a model primate subject. The gibbon has been used for HBV transmission studies in our laboratory in the past and has been shown to be susceptible to HBV infection (86).

METHODS: Mayo, a chronic Hepatitis B surface antigen (subtype adw) carrier, has been implicated as the source for two cases of Hepatitis B infection. His contact with these cases involved sexual activity. Mayo has provided semen (12 ml over a 48 hours period). Studies on semen collected at this time showed AUSRIA II P/N = 48. Six ml of this semen, frozen at -70°C, were forwarded from Dr. Harvey Alter for this study. Thawed semen showed AUSRIA IIP/N - 8.4.

Table 68 Gibbon Exposure Schedule

Gibbon	Age(yr)	Sex	Route of Semen Administration	Dose	Day	Total Dose
PC-21	3.0	F	Intra-vaginal	1.2 ml	D0, D1, D2	3.6 ml
PC-16	3.5	F	Subcutaneous	0.6 ml	D0, D1, D2	1.8 ml
PC-13	4.5	F	None (controls*)	-	-	-
PC-14	4.0	M				
B-40	11.0	M				
PC-26	2.5	F				

* All four gibbons carried anti-HB_s

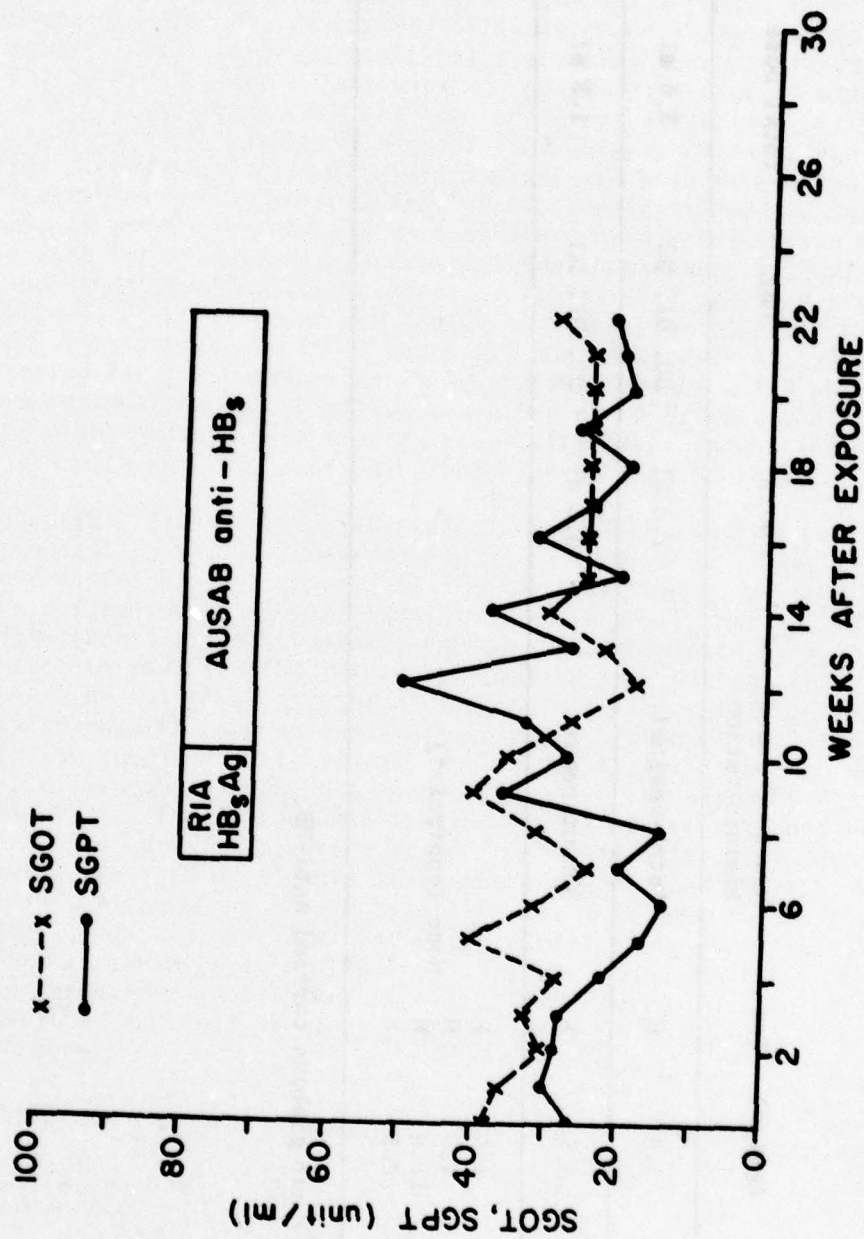


FIGURE 5. RESPONSE OF GIBBON PC-21 TO SEXUAL TRANSMISSION OF HUMAN SEMEN CONTAINING HB_sAg.

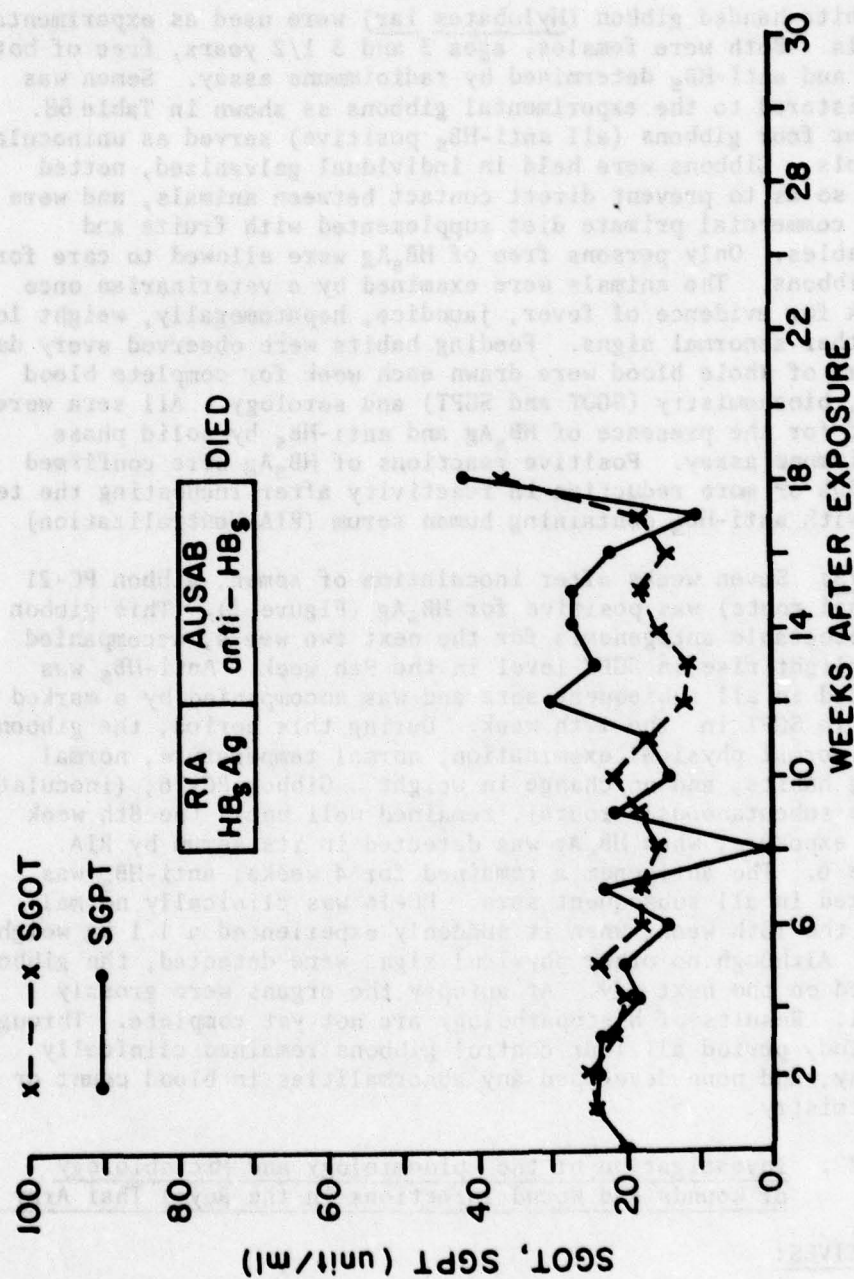


FIGURE 6. RESPONSE OF GIBBON PC-16 TO THE SUBCUTANEOUS INJECTION OF HUMAN SEMEN CONTAINING HB_sAg

Two white-handed gibbon (Hylobates lar) were used as experimental animals. Both were females, ages 3 and 3 1/2 years, free of both HB_sAg and anti-HB_s determined by radioimmune assay. Semen was administered to the experimental gibbons as shown in Table 68. Another four gibbons (all anti-HB_s positive) served as uninoculated controls. Gibbons were held in individual galvanized, netted cages so as to prevent direct contact between animals, and were fed a commercial primate diet supplemented with fruits and vegetables. Only persons free of HB_sAg were allowed to care for the gibbons. The animals were examined by a veterinarian once a week for evidence of fever, jaundice, hepatomegally, weight loss and other abnormal signs. Feeding habits were observed every day. Five ml of whole blood were drawn each week for complete blood count, biochemistry (SGOT and SGPT) and serology. All sera were tested for the presence of HB_sAg and anti-HB_s by solid phase radioimmune assay. Positive reactions of HB_sAg were confirmed by a 50% or more reduction in reactivity after incubating the test sera with anti-HB_s containing human serum (RIA-Neutralization).

RESULTS: Seven weeks after inoculation of semen, gibbon PC-21 (vaginal route) was positive for HB_sAg (Figure 5). This gibbon had detectable antigenemia for the next two weeks, accompanied by a slight rise in SGPT level in the 9th week. Anti-HB_s was detected in all subsequent sera and was accompanied by a marked rise in SGPT in the 12th week. During this period, the gibbon had a normal physical examination, normal temperature, normal eating habits, and no change in weight. Gibbon PC-16, (inoculated by the subcutaneously route), remained well until the 8th week after exposure, when HB_sAg was detected in its serum by RIA. Figure 6. The antigenemia remained for 4 weeks; anti-HB_s was detected in all subsequent sera. PC-16 was clinically normal until the 18th week, when it suddenly experienced a 1.1 kg weight loss. Although no other physical signs were detected, the gibbon expired on the next day. At autopsy the organs were grossly normal. Results of histopathology are not yet complete. Throughout the study period all four control gibbons remained clinically healthy, and none developed any abnormalities in blood count or biochemistry.

22. Investigation of the Epidemiology and Microbiology of Wounds and Wound Infections in the Royal Thai Army (RTA)

OBJECTIVES:

1. To describe the extent and distribution (by agent and anatomical site) of wounds, incurred as a result of combat, appearing at 2^o and 3^o surgical centers.

2. To establish the types and quantities of microbial flora coexisting with these injuries.

3. To relate microbial occurrence to risk of overt infection, severity and type of injury, subsequent treatment and residual morbidity.

4. To ascertain the value of the injury/microbiology/infection approach for use in predicting infection potential in combat injuries.

BACKGROUND: Descriptions of wounds incurred and microbiologic flora associated with those wounds have been made in the English language literature for each major military excursion since World War II. Primarily by American clinicians, these accounts have suffered from two basic deficiencies unrelated to the expertise of the authors. The primary problem has been that chains of evacuation, while efficient, have been so long that no one person or group of persons could observe the effects of primary microbiological inoculation into a combat injury and its subsequent effects related to morbidity.

The second corollary problem is that microbiologic data have been reported as occurrence of contamination of the wound site at the time of injury or as occurrence of overt infection later in the post-surgical period without a relationship being established between them.

Pre-injury antibiotic prophylaxis in RTA troops is confined to a sulfadoxine-pyrimethamine combination for antimalarial prophylaxis. Post-injury antibiotic coverage is at the discretion of the treating physician, but usually includes ampicillin (personal communication, MAJ Narong Rodwanna).

The chain of evacuation of a wounded RTA soldier includes:

Emergency life-saving care-company-aidman
1^o surgical care - battalion aid station/brigade dispensary
2^o surgical care - provincial hospital/area military hospital
3^o surgical care - RTA hospital Bangkok
Convalescence-rehabilitation center Bangkok

Any or all of the above may be by-passed if the condition of the patient warrants it. Ninety percent of the cases seen at RTA hospital, Bangkok had received previous care at a provincial

Table 69.

ORGANISM	ANATOMICAL LOCATION OF INJURY												TOTAL	REMARKS			
	HEAD & NECK			CHEST & BACK			ABDOMEN			UPPER EXTREMITIES					LOWER EXTREMITIES		
	B	M	R	B	M	R	B	M	R	B	M	R			B	M	R
1. <i>Bacillus</i> spp.																1	Agent of injury
2. <i>Clostridium glycolicum</i>																1	B = Missile projectile
3. <i>Diphtheroid</i> spp	1															1	
4. <i>Enterobacter</i> spp.																7	M = Land mine
<i>E. aerogenes</i>																3	R = Rifle bullet
<i>E. agglomerans</i>																2	
<i>E. cloacae</i>																1	
<i>E. hafnia</i>																1	
5. <i>Escherichia coli</i>	1															2	
6. <i>Hemophilus parainfluenza</i>	1															1	
7. <i>Herella</i> spp										1						2	
8. <i>Pseudomonas</i> spp.																13	
<i>Ps. aeruginosa</i>																12	
<i>Ps. unspicied</i>	1															1	
9. <i>Staphylococcus</i> spp.																5	
<i>S. aureus</i>																1	
<i>S. epidermidis</i>																4	
10. <i>Streptococcus</i> spp.																3	
<i>S. faecalis</i>	1															1	
β																1	
<i>B.</i> (not group A)																1	
11. <i>Candida</i> spp.																1	
TOTAL ISOLATIONS	7	1	1	3			1				2	1	15	4		37	

hospital. Ten percent were either evacuated from an area military hospital or flown directly to Bangkok. (Personal communication, COL Boonket Loovanich).

METHODS: Microbiological Sample Collection: Both aerobic and anaerobic specimens were routinely collected from the wound site and a blood culture was taken concurrently. Wound specimens consist of tissue, fluid exudate or swab from infection surface, and are collected on admission, at surgery, and when clinically indicated. Cultures are inoculated into transport media and a blood culture bottle. Wound cultures are reported only when flora speciation and antibiotic sensitivity data are available. Blood specimens for culture are taken for culture on three successive days following any clinical indication of septicemia, following surgery, and following any positive culture report, and also taken if no specimen has been otherwise required for 3 days. Blood cultures are incubated for a minimum of 72 hrs., read at 24, 48 and 72 hrs., and then submitted to the main laboratory for further study. Any positive bottle culture is gram stained and examined microscopically prior to subculture.

Epidemiologic Data Collection: Each combat injured soldier is interviewed by a nurse as soon after admission as feasible. The interview concerns demographic data on the patient, type of injuring agent, if known (booby trap, mine, shoulder weapon, hand weapon, etc.), geographical location of the area in which the injury was sustained and activities at the time of injury. The nurse makes an objective assessment of injury severity using a standard scale. From the patient's chart, a clinical description of all injuries and concurrent medical problems (diabetes, helminthiasis etc.), therapy prior to admission, initial laboratory findings, and place and length of prior hospitalizations with this injury are abstracted.

RESULTS: A pilot study and training period is currently being performed at Phramongkutklao Hospital, Bangkok (RTA Hospital). Preliminary results on the first twenty-five patients enrolled are presented below:

Military status:	Enlisted (included 3 police)	20
	PVT	12
	CPL	2
	SGT	4
	MSG	2
	Civilian (working with military units)	4

Agent of injury:	Missile projectile	7
	Land mine	8
	Rifle bullet	10
Anatomical location of injury:	Head and neck	6
	Chest and back	3
	Abdomen	1
	Upper extremities	10
	Lower extremities	18
(Total exceeds 25 because of multiple site involvement)		

Casualties occurred on the Malaysian, Laotian and Cambodian borders as well as internally in the Northeast area of the country. The majority of injuries were incurred in inter-camp movement and patrolling.

From wound cultures, 37 isolates of 17 different species were made, but the largest single group, accounting for one-third of total isolates, were species of the genus *Pseudomonas*. Twelve of the 13 isolates were *P. aeruginosa* and one could not be speciated. No bacterial growth was observed in only five (20%) of the battle-field casualties cultured. The list of isolates obtained is presented in Table 69. No isolates were obtained from blood culture.

It is anticipated that this study will be continued at Phramongkutklao Hospital and at an area military hospital outside of Bangkok beginning on or about 1 October 1978.

23. Epidemiological and Ecological Studies of Scrub Typhus in Royal Thai Army Field Training Facilities

OBJECTIVES:

1. To prospectively determine the susceptibility and the exposure risk of Royal Thai Army personnel to *Rickettsia tsutsugamushi* during field training exercises.
2. To determine the prevalence of *Rickettsia tsutsugamushi* in selected species of small mammals and chiggers from areas and habitats utilized by troops during training.
3. To determine if there is a seasonal effect influencing the susceptibility risk of Royal Thai Army personnel.

4. To evaluate the use of regional, habitat and seasonal data for predicting human exposure and risk potential to scrub typhus in Thailand.

BACKGROUND: Outbreaks of scrub typhus in the Royal Thai Army have been isolated epidemic events. On the basis of hospitalized cases, personnel at greatest risk appear to be those undergoing primary field training in the central or Korat plateau areas as those in security forces deployed in the field (Sangkasuwan - personal communication). Sangkasuwan et al. (87) found R. tsutsugamushi in every area sampled in Thailand and also concluded that there was seasonality in the frequency of rickettsial isolations from chiggers and mammals. A similar seasonal pattern has been observed in human cases in Thailand (88).

The ecological complexity of Thailand offers unique opportunities for comparative studies to assess the influence of various ecological factors on the presence, prevalence and risk of R. tsutsugamushi to man. Recent development of a serological test for R. tsutsugamushi (89), more reliable than the standard Weil-Felix test, now provides a good basis for field studies of scrub typhus in Thailand.

METHODS: As a pilot project, class 1-4, 138 soldiers undergoing Special Forces training at Pak Chong, Nakhon Ratchasima province, contributed blood samples before and after their training. Training consisted of four weeks of mixed classroom and field problems and four days of jungle bivouac approximately 15-20 km. from the base camp. The troop training includes daily contact with forested or grassy areas and a bivouac in similar areas. Total field exposure was calculated for each individual and background information on age, rank, occupation (both in and outside of the military), home, travel and previous medical history was taken. The latter of the two blood samples was collected two weeks after the end of training. The blood samples were tested by the Weil-Felix (90) and by the indirect immunofluorescence tests (89).

Rodents and other small mammals were live-trapped in each habitat associated with troop training. Fifty to 100 traps were placed in 2-3 habitats each night. All small mammals collected were identified to species, sexed, aged, bled via cardiac or retro-orbital puncture and examined for ectoparasites. Rodent rickettsial identification will be done at AFRIMS by the IFA technique (89). Attached chiggers were gently scraped off and placed in vials containing 70% ETOH. Chiggers were counted and collated according

Table 70. Small Mammals Collected, by Habitat, in the Pak Chong Special Forces Camp Training Areas, 18-27 June 1978

Habitat	Trap Night	Mammals								
		<u>Bandicota</u> <u>salvilei</u>	<u>Menetes</u> <u>berdmorei</u>	<u>Rattus</u> <u>bukit bukit</u>	<u>Rattus</u> <u>losea</u>	<u>Rattus</u> <u>rattus</u>	<u>Rattus</u> <u>sabanus</u>	<u>Rattus</u> <u>surifer</u>	<u>Tupaia</u> <u>glis</u>	Total
Early Regenerating Grass	760	1	-	2	4	63	1	4	4	79
Early Regenerating Evergreen	216	-	-	-	-	5	-	7	-	12
Secondary Evergreen	858	-	2	3	-	11	2	7	16	41
Total	1,834	1	2	5	4	79	3	18	20	132

Table 71. Small Mammals Collected, by Habitat, in the Royal Thai Army Bivouac Area in Ban Wang See Sod, 17-26 July 1978.

Habitat	Trap Nights	Mammals					Total
		Bandicota indica	Mus cervicolor	Rattus bukit bukit	Rattus	Tupaia glis	
Early Regenerating Grass (Lalang)	792	1	6	1	73	1	82
Secondary Evergreen	329	-	-	-	10	6	16
Banana Orchard	44	-	-	-	1	1	2
Total	1,165	1	6	1	84	8	100

Table 72. Chiggers and Rickettsia tsutsugamushi Isolations
from Pak Chong, Royal Thai Army Training Center

Chigger species	PAK CHONG						Total	
	S.F. Camp		Ban Wang See Sod					
	Number screened	Chiggers Infected No.	%	Number screened	Chiggers Infected No.	%	Number screened	Chiggers Infected No. %
<u>L. (L.) deliense</u>	144	9	6.25	18	-	-	162	9 5.56
<u>L. (L.) miculum arvinum</u>	23	3	13.04	-	-	-	23	3 13.04
<u>L. (L.) species C</u>	-	-	-	1	-	-	1	- -
<u>L. (Trom.) paniculatum</u>	1	-	-	-	-	-	1	- -
<u>Ascho. (Lau.) indica</u>	-	-	-	1	-	-	1	- -
Total	168	12	7.14	20	-	-	188	12 6.38

to host species and habitat. Unengorged chiggers were collected in the troop training areas by black plates. Chiggers collected from black plates were kept alive in vials of water and sent to USAMRU-Kuala Lumpur, for rickettsia isolation by the micro direct fluorescent antibody (MDFA) technique (91).

RESULTS: The initial part of the study involved a thorough reconnaissance of the two study areas. The terrain surrounding the base camp is government owned and is hilly and primarily covered with secondary evergreen forests, although disturbed areas with early regenerating vegetation (woody plants, grass) are also found in several of the training areas. The government owned property is completely surrounded by privately owned rolling hills which have been cleared of forest and are planted in agricultural crops, primarily maize. Several training areas utilized by the troops are on the edge of the government property and adjacent to the maize fields. The soil in the base camp training areas is primarily red-orange clays. The forests in the area are fairly well preserved with considerable humus and leaf litter, indicating that fires are uncommon.

The troop bivouac areas are located on non-government property in a valley at the foot of forested mountains. These sites consist primarily of grass (lalang) and isolated patches of regenerating and secondary evergreen forest. The bivouac areas are usually located in between or next to private agricultural property. The civilians in the area annually burn the maize stalks and adjacent grass and forest areas. Consequently, there is little humus and leaf litter present. The soil type in the bivouac areas is a sandy loam which dries very quickly.

The first entomological-rodent trapping studies occurred during the period 18 June-26 July 1978, and were conducted during two separate 10 day periods. The first period, 18-27 June, corresponded with the first 10 days of troop training around the base camp. The second period, 19-26 July, corresponded with the troop bivouac period in a small village area, Ban Wang See Sod, about 15-20 km from the base camp.

During the first entomological study period three basic habitats in nine training areas were trapped for small mammals, i.e., early regenerating grass, early regenerating evergreen and secondary evergreen. A total of 132 small mammals of eight species were trapped in these habitats over the 10 day period (Table 70). Rattus rattus was the most commonly trapped mammal. A total of

21 collections (black plate) were made for unengorged chiggers. These collections resulted in 168 chiggers which were sent to USAMRU-Kuala Lumpur for rickettsia isolations. Of the 168, 7.2% of the specimens were found positive for Rickettsia tsutsugamushi (Table 71). The infected specimens included nine Leptotrombidium (L) deliense and three Leptotrombicium (L) miculum arvinum. During the second entomological study period three basic habitats in and adjacent to two bivouac areas were trapped, i.e. early regenerating grass (lalang), secondary evergreen and banana orchard. A total of 100 small mammals of five species were captured in these habitats over the 10 day period (Table 72). Again, R. rattus was by far the most commonly trapped mammal. A total of 14 black plate collections were made in the areas for unengorged chiggers, resulting in only 20 specimens. These specimens were sent to USAMRU-Kuala Lumpur and all were found negative for R. tsutsugamushi (Table 73). The low chigger densities on the black plates in this study area were probably due to the low leaf litter-humus content on/in the sandy-porous soil. Although rain fell nearly every day and chiggers were very abundant on the trapped mammals, the disturbed nature of the soil and vegetation was apparently not conducive to high chigger densities in the absence of the mammals or their burrows. Of 138 soldiers, none showed Weil-Felix titers suggestive of recent infection (160) in the pre-training serum sample. No specimens had an IFA titer of 1:50 or greater. Post-training specimens demonstrated a four fold or greater titer rise in six soldiers by the Weil-Felix examination (all were titer rises from 1:20 to 1:40), but all soldiers were negative by the IFA technique.

The rodent sera from both study areas remain to be examined for R. tsutsugamushi antibody levels. Engorged chiggers taken from the mammals captured during both study periods are being mounted on slides for later identification.

24. The Prevalence of Poliovirus Infection in a Lower Socioeconomic School Population

OBJECTIVE: To define the prevalence of poliovirus antibodies in a school population in a socioeconomic environment in Bangkok.

BACKGROUND: Improved sanitation is a key element of the public health program of a developing country. As a consequence, one might predict that naturally-acquired infections with polioviruses in early childhood would become less prevalent as living conditions improved. A possible risk of postponed exposure to wild polio-

Table 73. Age Specific Prevalence of Polio Antibody in School Children of Lower Socioeconomic Status, 1977.

Age (yrs)	No. Tested	No. and % of Positive* Individuals Against						All three types	
		Polio I		Polio II		Polio III		No.	%
		No.	%	No.	%	No.	%		
4	60	43	71.1	47	78.3	39	65	27	45
5	66	51	77.3	61	92.4	44	66.7	37	56.
6	91	76	83.5	86	94.5	82	90.1	68	74.7
7	188	169	89.9	187	99.5	172	91.5	156	83
8	199	184	92.5	194	97.5	165	82.9	151	75.91
9	227	215	94.7	223	98.2	208	91.6	200	88.1
10	258	248	96.1	248	96.1	237	91.9	225	87.2
11	211	198	93.9	203	96.2	185	87.7	173	81.99
12	243	238	97.9	241	99	225	92.6	217	89.3
13	259	247	95.4	253	97.7	242	93.4	232	89.6
14	134	130	97.0	132	98.5	123	91.7	119	88.8
15	53	51	96.2	52	98.1	49	98.1	49	92.4

Total	1989	1850	93.0	1927	96.88	1774	89.19	1654	83.16
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* Positive neutralization antibody for dilution 1:5

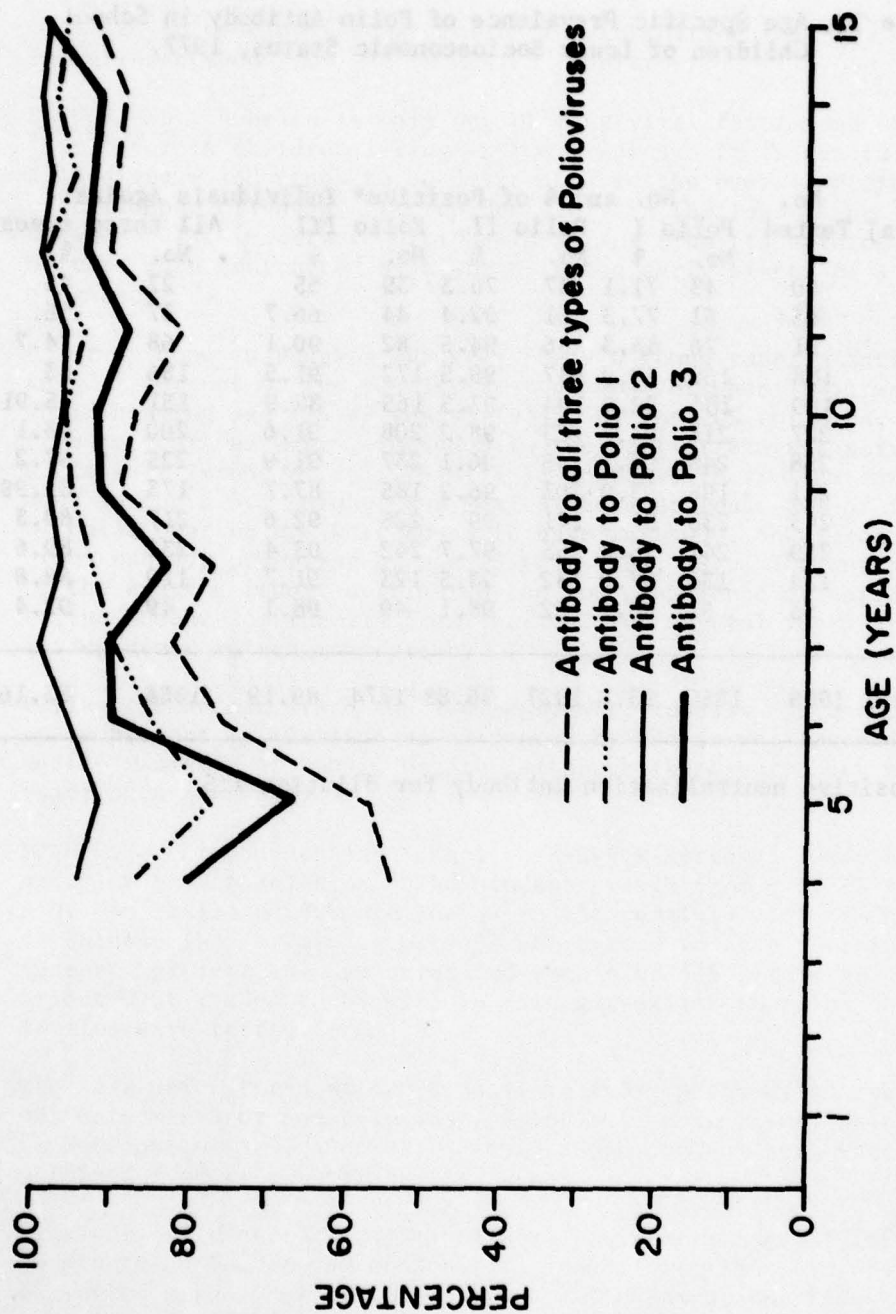


FIGURE 7. AGE DISTRIBUTION OF NEUTRALIZING ANTIBODY TO THREE TYPES OF POLIO VIRUSES, PIBUL PRACHASAN SCHOOL. 1977-78

viruses is an increase in apparent, paralytic poliomyelitis cases. As the cost of vaccination of large groups may be too great for countries with limited economic resources, periodic studies to evaluate the immune status to poliovirus among children may be very useful in public health planning.

METHODS: Sera were obtained from volunteer students of the Pibulprachasan School in Din Daeng area of Bangkok. A questionnaire requesting the student's past history of immunization was completed by the parents of participating students. Metabolic inhibition tests for detection of the presence of neutralizing antibody to the three types of polioviruses were performed. The method of detection is briefly as follows: heat-inactivated sera were diluted to 1:5 and 1:10; 0.05 ml of diluted sera were mixed with an equal volume of 100 TCID₅₀ of either Polio I, Polio II, or Polio III virus in suspension in sterilized microtiter plates. The virus-serum mixtures were incubated at room temperature for 30 minutes; then 0.05 ml of a Hela cell suspension (100,000 cell/ml) was added and covered with mineral oil. Plates were incubated in a 35°C incubator and results read at 7 to 8 days. The inhibition of cell metabolism, as shown by a red color of the Phenol red PH indicator in the medium, indicated lack of Poliovirus antibody. A neutralizing antibody titer of less than 1:5 was interpreted as negative.

RESULTS: Forty-five percent of the 4-year old school children studied had serum neutralizing antibodies against all three types of polioviruses. By age nine, almost ninety percent had antibody to all three types of polioviruses (Table 73 and Figure 7). Of the total of 1989 students, 83% had had exposure to poliomyelitis viruses of all three types. Information collected by questionnaire suggested that most had never received polio vaccine. By comparing the present study to a similar study of Thai children in 1961 (92), when 50% of study population had antibody to all three types of polioviruses at the age of four, it appears that factors influencing exposure to polioviruses have not significantly changed. Children continue to be exposed to polioviruses early in life, and the probability of paralytic poliomyelitis continues to be relatively low.

25. Rubella Antibody Prevalence in Bangkok School Children

OBJECTIVES:

1. To determine the pattern of distribution of rubella antibody in school children in Bangkok.

2. To develop a hemagglutination-inhibition test for rubella antibody for use in AFRIMS Laboratory.

BACKGROUND: Rubella is only one of many viral fever-rash syndromes that occur in children living in the tropics. It is generally a mild disease and tends to be discounted in the overall picture of childhood diseases. Its epidemiology in Thailand is not well defined. The importance of rubella arises from its teratogenic effects when contracted by a woman in the early stages of a pregnancy.

A survey done in Bangkok during 1969-1970 found rubella antibody in 52.7 percent of the females tested, most of whom (84%) were of child bearing age (15-45 years) (93). Of the 205 children, ages five to fourteen years, included in the earlier Bangkok survey 53.2% were seronegative. This is in contrast to similar surveys done in the U.S., Europe, and Mexico where at least 80% of females of child bearing age were found to have antibody, the seroconversions usually occurring by age 15 years (94, 95). The authors of the Bangkok report attributed the low prevalence of antibody to the absence of rubella from Thailand for several years preceding its epidemic return in September 1967.

During the decade since the 1967 epidemic, rubella has continued to be present in Thailand as evidenced by the surveillance data of the Ministry of Public Health, although there have been no major outbreaks (96).

METHODS: Phibunprachasan School in Bangkok serves a lower income and slum area population. The students range from 4 to 15 years old. An estimated 82% are Thai with the remaining 18% of Chinese or Chinese-Thai origins. Of 1987 who agreed to take part in a research project, an age stratified sample of 528 sera were selected at random to be used in this survey. Both males and females were tested.

The sera were stored at -20°C until tested. Antibody to rubella was determined by hemagglutination-inhibition test (HI) using microtechniques (97, 98, 99). Antigen and control sera were obtained from Flow Laboratories, U.S.A.

Pretreatment of sera: 0.1 ml of sera was inactivated at 56°C for 30 minutes. To remove nonspecific inhibitors, the sera was extracted with 10 ml of cold acetone. The sample was then centrifuged (1800 rpm, 7-10 min.) and the supernate discarded.

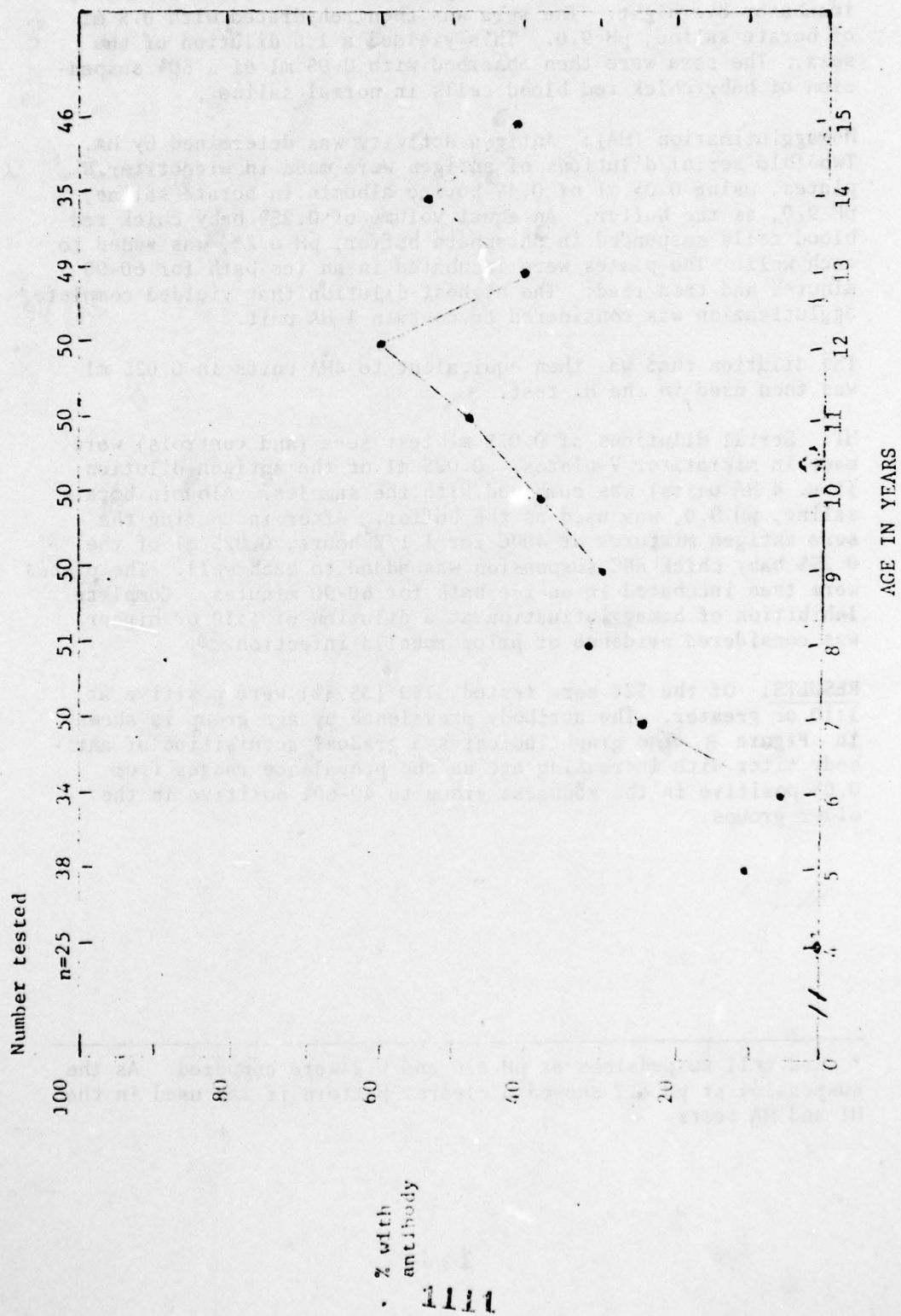


FIGURE 8. Prevalence of III Antibody to Rubella in Bangkok School Children

After the second extraction the precipitate was dried in a 37°C incubator overnight. The sera was then rehydrated with 0.5 ml of borate saline, pH 9.0. This yielded a 1:5 dilution of the sera. The sera were then absorbed with 0.05 ml of a 50% suspension of baby chick red blood cells in normal saline.

Hemagglutination (HA): Antigen activity was determined by HA. Two fold serial dilutions of antigen were made in microtiter U plates, using 0.05 ml of 0.4% bovine albumin in borate saline, pH 9.0, as the buffer. An equal volume of 0.25% baby chick red blood cells suspended in phosphate buffer, pH 6.2*, was added to each well. The plates were incubated in an ice bath for 60-90 minutes and then read. The highest dilution that yielded complete agglutination was considered to contain 1 HA unit.

The dilution that was then equivalent to 4HA units in 0.025 ml was then used in the HI test.

HI: Serial dilutions of 0.025 ml test sera (and controls) were made in microtiter V-plates. 0.025 ml of the antigen dilution (i.e. 4 HA units) was combined with the samples. Albumin borate saline, pH 9.0, was used as the buffer. After incubating the sera antigen mixtures at 40°C for 1 1/2 hours, 0.025 ml of the 0.25% baby chick RBC suspension was added to each well. The plates were then incubated in an ice bath for 60-90 minutes. Complete inhibition of hemagglutination at a dilution of 1:10 or higher was considered evidence of prior rubella infection.

RESULTS: Of the 528 sera tested, 189 (35.8%) were positive at 1:10 or greater. The antibody prevalence by age group is shown in Figure 8. The graph indicates a gradual acquisition of antibody titer with increasing age as the prevalence ranges from 0.0% positive in the youngest group to 40-60% positive in the older groups.

* Red cell suspensions at pH 6.0 and 6.2 were compared. As the suspension at pH 6.2 showed a clearer pattern it was used in the HI and HA tests.

26. Chemotherapy of Gnathostomiasis

OBJECTIVE: To continue to search for chemicals with effective chemotherapeutic activity against advanced third-stage larvae of Gnathostoma spinigerum in experimentally infected mice and cats.

BACKGROUND: These studies are a continuation of the work reported in previous years. Many anthelmintic drugs have been evaluated for possible chemotherapeutic activity against experimental G. spinigerum infection of white mice with advanced third-stage larvae or migrating stages of the worm. All drugs tested have been ineffective except Ancylosol disphenol given in subcutaneous injections at one week intervals to infected white mice, which showed significantly reduced numbers of G. spinigerum larvae compared with control mice. However, a second group of infected white mice showed no significant reduction of the larvae after being injected, also with 8 doses of the same drug (100). The screening test with this drug on the mice infected with the G. spinigerum larvae was then suspended.

It was also shown previously (100) that 12 doses of 0.05 ml/lb body weight of parenteral Ancylosol given to 2 cats at 10 day intervals were very effective in reducing numbers of G. spinigerum larvae; the dosage of this drug at 0.04 ml/lb body weight remained to be tested.

Etrenol (Hycanthone) was previously tested on mice infected with G. spinigerum advanced third-stage larvae by oral administration but showed no therapeutic effect on the infection (101).

METHODS: Ancylosol: Two adult domestic cats, after being kept at the Veterinary Medicine facility for about one year and proved negative for natural Gnathostoma infection (monthly stool examination for Gnathostoma eggs by Formalin Ether Sedimentation technique (Ritchie)) were infected each with 71 and 99 G. spinigerum advanced third-stage larvae obtained from experimentally infected mice. After the infection was permitted to continue for 37 and 42 days, each cat was administered, parenterally, 12 doses of 0.04 ml per lb body weight at 10-day intervals.

Mice of the ICR strain were infected by oral administration, each with five G. spinigerum advanced third-stage larvae. After the infection became established, the following drugs and regimens were tested: 1. Etrenol (Hycanthone Winthrop Products Inc.): This drug is used for schistosomicidal activity. The drug was given by intramuscular injection and the dosage guide for treating

bilharziasis was followed. 2. Trodax: (Nitroxynil aqueous solution of the eglumine salt for subcutaneous administration) proved to give therapeutic results against fascioliasis of sheep and cattle and against *Ancylostoma caninum* in dogs. This is produced by May and Baker LTD., England. 3. Joint (Oral administration) or phenylene-diisothiocyanate-(1,4), proved to give therapeutic results on nematodes and cestodes infesting a variety of animals. The drug is produced by Farbwerke Hoechst AG, Germany.

Infected control mice for every drug tested were injected only with sterile distilled water. After completion of the treatment schedule, all mice were sacrificed and necropsied. Parasites found in various tissue were counted and the results recorded.

RESULTS: The two cats treated with 12 doses of Ancylo1 0.04 ml/lb body weight at 10-day intervals showed an effective therapeutic results, as one was negative and the other had only two living larvae in the diaphragm, compared with 16 larvae and immatures found in the two control cats (Table 74. The treated animals showed no gross pathological changes or toxic effects of the organs caused by the drug. It is suggested that Ancylo1 be tested parenterally in non-human primates infected with migrating larval stage of *G. spinigerum*, using the same regimen of multiple dosages of the drug and longer intervals between the doses. The results obtained from the experiment on primates should lead to consideration of further trials, on man if possible.

Etrenol (Hycanthone): This drug was given intramuscularly to the infected mice in one and two dose regimens (one daily dose) of 5 mg, 10 mg, 15 mg, 20 mg per/kg body weight. The results with these small dosages are shown in Table 75. There was no significant reduction in the number of gnathostome larvae in the treated mice compared with the control mice for any regimen. Therefore, Etrenol (Hycanthone) given intramuscularly at these lower dosages is considered to have no therapeutic effect. Further trials on the infected mice at larger dosages of the drug, especially with 50 mg, 100 mg, 150 mg, per kg body weight given intramuscularly, are now in progress.

Jonit: This drug was administered orally one dose for one day and one daily dose for two days using dosages of 50, 100, 150 mg/kg body weight on mice infected with *G. spinigerum* advanced third-stage larvae. The results are shown in Table 76. The drug is considered to have no therapeutic value in the treatment of *G. spinigerum* infection.

Trodax (Nitroxynil): One dose of the drug was administered subcutaneously using doses of 5, 10, 15 and 20 mg/kg body weight on mice infected with G. spinigerum advanced third-stage larvae. The results are shown in Table 77. The drug is considered to have no therapeutic value in the treatment of G. spinigerum infection.

SUMMARY: Two cats infected with G. spinigerum migrating stage larvae were successfully treated with 12 doses of Ancyrol 0.04 ml/lb body weight at 10-day intervals. Neither cat showed any evidence of gross pathological changes caused by the drug. It is suggested that Ancyrol be tried on primates with the same regimen before trials be begun in man. Intramuscular administration of Etranol (Hycanthone) to infected mice with small dosages was ineffective and further trials at larger dosages are in progress.

Trodax (Nitroxynil) subcutaneous administration and oral administration of Joint (Phenylene-diisothiocynate-(1,4) were ineffective in the chemotherapy of Gnathostoma spinigerum in experimentally infected mice.

This is a final report from this Laboratory. Work in progress will be continued at the Faculty of Tropical Medicine, Mahidol University.

Table 74. Treatment of *Gnathostoma spinigerum* Migrating Stage Larvae Infected Cats with Multiple Subcutaneous Doses of Ancylool Disophenol, 0.04 ml per lb Body Weight per Dose for 12 Doses at 10-day Intervals

Drug Dose (mg/kg)	Total Doses of Drug to Each Cat	Number of Cat Treated	Number of Third-stage Larvae Given to Each Cat	Age of The Worm in Cat Before Treatment (in day)	Autopsy Finding		Remarks
					Worm Survival Rate %	Number and Stage of Living Worm & Infected Organs	
0.04	12	2	77,99	37,42	0,2.02	0, 2 Living larvae in diaphragm	1 Dead larva in diaphragm
-	-	2	50	-	16 each	8 Living larvae in muscles and liver 8 immatures in muscles, stomach wall and diaphragm	* 2 Control cats

* Results obtained from the Annual Progress Report April 1976 - September 1977.

Table 75. Treatment of Gnathostoma spinigerum Advanced Third-stage Larvae Infected Mice each with One Dose and Two Doses (One Daily Dose) of Etenol (Hycanthone) Intra-muscular Injection

Hycanthone Drug Dose (mg/kg)	No. of Drug Dose	No Mice Infected with the Larvae	Total Larvae administered to the Mice	Necropsy Findings		Time of Necropsy (Days)*
				Total Larvae (%)	Organ Found Infected	
5	1	20	100	41(41)	Livers, muscles	22-36
5	2	5	25	11(44)	Livers, muscles	22-36
Control	-	10	50	21(42)	Livers, muscles	12-36
10	1	20	100	43(43)	Livers, muscles	40-41
10	2	5	25	12(48)	Muscles	40-41
Control	-	10	50	23(46)	Livers, muscles	35-42
15	1	20	100	49(49)	Livers, muscles	6-49
15	2	5	25	12(48)	Muscles	6-49
Control	-	10	50	26(52)	Livers, muscles	6-51
20	1	20	100	48(48)	Livers, muscles	20-50
20	2	5	25	12(48)	Muscles	20-50
Control	-	10	50	25(50)	Livers, muscles	27-51

* Days after administration of last drug dose.

Table 76. Treatment of *Gnathostoma spinigerum* Infected Mice with Jonit Oral Administration for One and Two Day Courses

Jonit Drug Dose (mg/kg)	No. of Infected Mice Treated		Third-stage Larvae Found After One Dose Number (%)	Third-stage Larvae Found After Two Doses Number (%)	Time of Necropsy (Days)*
	One Dose	Two Doses			
50	10	10	24(48)	24(48)	23
100	10	10	25(50)	25(50)	21
150	10	10	26(52)	24(48)	8-35
Control	20	20	47(47)	47(47)	21-37

* Days after administration of the drug.

Table 77. Treatment of Gnathostoma spinigerum Infected Mice with One Subcutaneous Dose of Trodax (Nitroxynil)

Trodax (Nitroxynil basis) Drug Dose (mg/kg)	No. of Infected Mice Treated	Third-stage Larvae Found, Number (%)	Time of Necropsy (Days)*
5	20	46(46)	22
Control	10	25(50)	22
10	20	51(51)	20
Control	10	23(46)	20
15	20	48(48)	21
Control	10	24(48)	21
20	20	42(42)	23
Control	10	25(50)	23

* Days after treatment

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Work Unit 008 Tropical and Subtropical Diseases in Military
Medicine (AFRIMS)

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1. Snitbhan, R., Scott, R.M., Bancroft, W.H., Karwacki, J.J., Manomuth, C. Evaluation of Serological Tests for Detecting and Identifying Markers of Hepatitis B Virus Infection. Proceedings of the 18th SEAMEO-TROPMED Seminar, August 1977, Kuala Lumpur, Malaysia.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION#	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6525	78 10 01	DD-DR&E(AR)636	
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c. CONTRIBUTING		CARDS 114F					
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ADDRESS: Washington, D. C. 20012				Brasilia			
				Brasilia, Brazil			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide DOD Security Classification Code)			
NAME: RAPMUND, Garrison, COL				NAME: RADKE, M. G. LTC			
TELEPHONE: 202-576-3551				TELEPHONE: 272-4548 (Brazil)			
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Foreign intelligence not considered				NAME: PRATA, Aluizio, MD			
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29. TECHNICAL OBJECTIVE (34. APPROACH, 35. PROGRESS) (Provide individual paragraphs identified by number, precede text of each with Security Classification Code)							
<p>23. (U) Find new prophylactic and curative drugs which can be used to prevent and cure schistosomiasis infections that would be acquired by U. S. military and DOD civilians in the event of deployment in endemic areas such as South America, Caribbean, Africa, Middle East and Far East.</p> <p>24. (U) The WRAIR Anti-Schistosomal Drug Development Program submits drugs for prophylactic and therapeutic testing against schistosomiasis mansoni in mice. The prophylactic mortality test screens drugs against mice exposed to 3,000 or more S. mansoni cercariae, and the drugs are given subcutaneously in a single dose at 640 mqs/kg. The curative test uses mice exposed to 160 cercariae, and 30 - 35 days later drugs are given subcutaneously at 100 mqs/kg for five days. Prophylactic drug activity is measured by mouse survival and curative drug activity is measured by sick and dead worms in the liver. During the reporting period, we initiated experiments aimed at expanding the program to include a Secondary Curative Test.</p> <p>25. (U) 77 10 - 78 09. This research is complementary to studies being conducted under DAOB 6525, Work Unit 086, entitled "Chemotherapeutic Studies on Schistosomiasis". The laboratory Biomphalaria glabrata (Paulista) snail colony maintained an average population of 1,000 - 1,200 cercariae shedding snails. The exposed snail survival rate was 88 percent, with a 54 percent infection rate. Thus 3 to 5 million cercariae were available for weekly mouse exposures. During FY78, 1,195 selected WRAIR Bottle Number drugs were tested for prophylactic and curative activity. The results were as follows: A. Prophylactic testing: 396 drugs negative or unconfirmed active, 136 drugs toxic, and 6 drugs confirmed active; B. Curative testing: 610 drugs negative or unconfirmed active, 32 drugs toxic, and 15 drugs confirmed active; C. 5 drugs were active in both test systems. For technical report see Walter Reed Army Institute of Research Annual Report, 1 Oct 77 - 30 Sep 78.</p>							

DD FORM 1498
1 MAR 66

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

PROJECT NO: 3M162770A802 MILITARY PREVENTIVE MEDICINE

WORK UNIT: 009 ANTI-SCHISTOSOMAL DRUG DEVELOPMENT

DESCRIPTION:

Schistosomiasis continues to be ranked among the most important of the tropical diseases, yet we still lack suitable means for chemotherapeutic management, and have no means for chemoprophylactic management. The only drugs currently available demonstrate only partial curative efficacy and are often accompanied by adverse side effects ranging from carcinogenicity to headaches and dizziness. Considering the actual and potential global commitments of United States military and civilian personnel, the risks to infection with one of the human schistosomes remains high. Indeed the incidence of infection within foci of local indigenous populations may approach 100 percent. Consequently, a major research effort in anti-schistosome drug development is being carried out by the Walter Reed Army Institute of Research in conjunction with the University of Brasilia (USAMRU-Brasilia). The test compounds are obtained from the Division of Experimental Therapeutics (WRAIR) and are tested for prophylactic and curative activity in mice at the USAMRU-Brasilia. Our ultimate objectives are the identification of compounds with a high potential for use in the prevention and treatment of schistosomiasis mansoni.

PROGRESS:

a. Laboratory Facility. During the reporting period the laboratory operations were improved by the addition of two new items of equipment: 1) a new Mettler electronic top loading analytical balance has considerably reduced the drug preparation time and improved the accuracy of the finished product; 2) the acquisition of a new stereozoom Olympus microscope has provided the increased capability for procedures requiring these features (e.g. liver squash preparations).

b. Animal Facilities. As reported earlier, the con-

version to corncob cellulose bedding effectively eliminated the problem of mouse infection failures; this favorable situation has continued throughout the current reporting period. Early in the year, serious problems were encountered at the Bioterio of the University of Brasilia relative to the weekly supply of healthy animals in the numbers required. While our normal laboratory operations require a full complement of 400 mice per week, weighing between 18 and 23 grams, on many weeks we received no mice at all or only a few mice. These were underweight and quite often sick. Later histopathologic analyses confirmed an almost universal infection with coccidiosis concurrent with multifocal hepatitis, the combination of which with schistosome infection often led to bacterial liver abscesses. Efforts are presently underway to upgrade the mouse breeding colony and to improve the environmental conditions under which the colony is maintained.

c. Snail Colony. The Biomphalaria glabrata (Paulista Strain) snail colony continues to provide Schistosoma mansoni cercariae in sufficient quantities to perform the weekly mouse exposures for drug testing and life cycle maintenance. A weekly average of 400 snails were exposed to miracidia recovered from macerated infected mouse livers. An acceptable level of prepatent mortality (22 percent) was obtained; of the survivors which were screened 42 days post-exposure, 54 percent were positive for emerging cercariae. These are maintained for future cercariae collections and a weekly average population of 1,000 to 1,200 positive snails are on hand at one time. In general approximately 45 percent of the snails exposed are later recovered with patent infections.

d. Drug Testing. A total of 1,244 bottle numbered drug samples were received from WRAIR during the reporting period. Of these, 703 compounds were designated for both prophylactic (PMT) and curative (PCT) testing; 539 compounds were designated for only curative testing; and two compounds were designated for only mortality testing. (See below for descriptions of the test systems) While these compounds were shipped and received during the year, many have not yet been tested because of our increased emphasis on the important matter of retesting older compounds which showed indications of activity or toxicity, or for which the control group data were questionable. The backlog of newer compounds is currently being diminished

as retesting is completed.

We have the capability of performing twice monthly tests in each of the two systems, prophylactic and curative. While normal PCT testing can evaluate 60 compounds at a time and PMT testing can evaluate 50 compounds at once, the mouse supply was the primary limiting factor. During the period of the above mentioned problems with the Bioterio mouse colony, we often received much reduced numbers in the weekly deliveries and on some weeks we received none. We are currently receiving approximately 75 percent of our full weekly complement of 400 mice (Swiss-Holland 40 albino, 45 ± 5 days old, weighing 18 - 23 grams). However, the test systems incorporate enough flexibility that testing can be continued using a reduced number of experimental compounds per test run. Additionally, the reduced numbers of animals provided the opportunity to perform other experimental evaluations (such as standardizations of a Secondary Curative Test) which required fewer numbers of animals. See Figure 1 which depicts the workload data for FY78.

e. Operating Personnel. The drug testing program is directed by one American Senior Investigator and supported by a staff of eight Brazilian Laboratory Assistants (one position vacant). The operating program is broken down into five work areas which are: 1) Snail Colony (two people); 2) Animal Service (two people); 3) Necropsy (one person); 4) Pharmacy (two people); and 5) Administration (one person). All individuals are cross-trained to perform the seven day work schedule of daily snail maintenance, subcutaneous and gavage drug administration, daily mouse maintenance with mortality checks, and mouse exposures to cercariae. Each individual is able to perform all duties in two other areas of work.

TEST PROCEDURES:

a. General. Whereas in past years, all drugs received were normally tested for prophylactic activity first, the current system now places a priority of prophylactic or curative testing on each compound. For those compounds received for testing in both systems, prophylactic testing is still performed first. All tests, prophylactic or curative, are performed with groups of five mice per drug per dosage schedule. all mice are individually tail-exposed 30 minutes to the numbers of cercariae required by the specific test. Drugs are routinely pre-

pared for administration in a peanut oil vehicle unless another vehicle (such as water, saline, alcohol, or cremophor) has been previously recommended. All drugs are administered subcutaneously unless orally (by gavage) has been designated. Likewise, all drugs are administered in terms of mgs per kg body weight of mouse recipient.

b. Primary Mortality Test (PMT). The PMT is a prophylactic test in that it evaluates drug activity against immature migrating larval schistosomes. Mice are exposed to 3,000 - 3,500 *S. mansoni* cercariae. Two days after exposure drugs are administered in a single inoculation to the five test animals per drug. The standard initial test dose is 640 mgs/kg and future testing repeats this dose with other groups at lower dosages. The 640 mgs/kg dose is a reduction of the 1280 mgs/kg dose used in earlier testing, but the experience of the past four years has indicated that nothing is gained by the higher dose in detecting active compounds, while the lower dose conserves the limited supply of compound for future testing.

For every PMT group there are control groups of 1) 50 infected untreated mice, 2) 10 normal mice, and 3) five mice treated with the reference drug Niridazole (640 mgs/kg). The infected untreated control mice will begin dying on day 20 post-exposure and none will survive past day 30 in most cases. Niridazole-treated mice survive until day 49. Active drugs are those for which treated mice survive two weeks after all infected control mice are dead. At 49 days, all surviving mice (controls and drug test) are perfused (Radke, et. al., 1961) for total worm burden determination. Drugs are considered toxic at the dosage given if recipient mice die within 10 days post-treatment (12 days post-exposure). All active or toxic compounds are scheduled for later retest confirmation at the same dosage and route of administration as the initial test. If positive confirmation is obtained for activity, then further testing at different dosages by both routes (subcutaneous or oral) is scheduled.

c. Primary Curative Test (PCT). The PCT is a curative test of a compound against an established *S. mansoni* infection in mice exposed to 160 cercariae. Thirty-three days post-exposure drugs (100 mgs/kg) are administered daily for five consecutive days (until day 37) in the same manner as described for the PMT. Three days following the last treatment, all mice are: 1)

killed individually by cervical dislocation; 2) the livers are immediately removed; 3) the livers are made into liver squash preparations; and 4) the numbers and condition of worms in the liver are determined for each surviving animal. Control groups for each PCT run are. 1) 20 untreated infection control mice; 2) 10 Niridazole treated mice (5 at 100 mgs/kg and 5 at 160 mgs/kg); and 3) five Oxamniquine treated mice (100 mgs/kg).

Criteria for drug activity are based upon the hepatic shift of adult worms from the mesenteries to the liver. This shift is presumed to be a result of drug pressure. Not only are the total numbers of liver worms determined but the conditions of those worms are also taken into consideration. The presence of dead worms is incontrovertible evidence of drug activity, while the presence of small, abnormally developed "sick" worms possessing little movement is evidence of possible activity requiring further testing.

Untreated control animals will normally show five to 15 worms in the liver and a test animal liver worm burden of 2 - 3 times that of the mean control liver worm burden is indicative of drug activity. Oxamniquine treatment produces high dead worm burdens in the livers of infected animals, while Niridazole normally produces high living (but sick) worm burdens at 100 mgs/kg and 160 mgs/kg, with the appearance of a few dead worms at the latter dose.

While the previous year we reported the PCT as in the developmental stages, we feel that we currently have sufficient experience with the test to place considerable confidence in the results as indicators of curative drug activity. The criteria for evaluation of results varies from those used in the earlier Japan program, primarily in terms of the numbers of worms in the livers of untreated control animals. But repeated testing has indicated higher normal liver worm burdens in our infections and the differences between the two programs can probably best be explained by snail/parasite strain and/or environmental variations. Our investigations on comparative strain differences produced inconclusive results. Additionally, we have incomplete data on the Japan program strain establishment upon which we can establish valid comparisons.

d. Secondary Curative Test (SCT). In an effort to

expand our drug testing capabilities, we have initiated the standardization of an SCT to complement the PCT. The rationale of this development rests in the requirement for following up on possibly active compounds by providing additional data on the duration of action of selected compounds over a period of time. It should be emphasized that the SCT is not a primary screen, as are the PCT and PMT. Rather it is intended as a more defined evaluation of drug efficacy and emanates from the primary test systems. Consequently, it is anticipated that considerably fewer drugs, and only those that show promise will be tested. The benefits will be a more complete understanding of test drug action upon which to base future testing.

The initial secondary test runs were organized to follow the method described by Bruce *et al* in the unpublished W.H.O. Document 73.30 ("Schistosomiasis Drug Test Systems"). That is, mice are exposed to 160 cercariae and treated with test compound daily for five consecutive days between days 33 and 37 post-exposure. Separate perfusions of the mesenteric, portal and hepatic circulations are then performed on days 7, 14, and 21 post-treatment. Our initial tests consisted of 150 mice divided among the following five groups (30 mice per group): 1) infected, no treatment; 2) treated with Niridazole, 100 mgs/kg; 3) treated with Niridazole, 160 mgs/kg; 4) treated with Niridazole 320 mgs/kg; and 5) treated with Oxamniquine, 100 mgs/kg. Ten mice from each group were then perfused at weekly intervals as mentioned above.

We soon learned several drawbacks to the described method. 1) The separate perfusions of liver, hepatic portal vein, and mesenteries are extremely time consuming and would necessitate the full time participation of at least three technicians for three days to complete one test run of controls and a reasonable number of test drugs. 2) Perfusion of livers is relatively meaningless, especially two and three weeks post-treatment, since dead worms within the hepatic circulation are almost impossible to dislodge and flush out. By the second week post-treatment, dead worm granulomas are already forming, as revealed in liver squash preparations performed after perfusion. Consequently, perfusion liver worm counts were lower than actually present and consisted only of living worms. 3) The exposure of mice to 160 cercariae

each (the same exposure dose as the PCT) created an undesirable mortality pattern among untreated control animals, in which 100 percent of these animals died prior to day 58 (three weeks post-treatment). In the PCT, this is of no concern since all animals are killed on day 40, prior to death from acute disease. But in the SCT it is necessary to obtain control animal survival through the third perfusion phase in order to obtain comparative worm burden determinations.

We are currently evaluating a modified SCT which consists of 1) exposure of mice to 100 cercariae (a sublethal dose for 58 days), 2) dividing each test and control group into two comparative subgroups, one for total perfusion and one for only liver squash preparations (as in the PCT), and 3) adding an additional perfusion/liver squash phase at three days post-treatment. These latter two modifications then bring the test more into line as a true secondary test by supplementing the previous primary test data under similar conditions. During the coming year we anticipate establishing the test as a standard procedure within the drug testing program.

OTHER SCHISTOSOMIASIS RESEARCH:

a. Snail Strain Studies. We last reported the differences in liver worm burdens between our PCT control data and that obtained earlier in the Japan drug testing program. A proposed reason for the abnormally high worm burdens in the Brazil program was the strain difference between the B. glabrata snail and/or schistosome maintained at the two laboratories. For comparative purposes, we obtained infected B. glabrata snails (Puerto Rican/Walter Reed Strain) from WRAIR (Kindly provided by CPT Lyford K. Greene, Department of Parasitology) and maintained them in the Brasilia laboratory. We exposed mice to cercariae collected from these snails and performed PCT and PMT tests on these different strain infections in parallel with routine test runs performed with our Paulista strain material. Both strain infections were treated identically in terms of cercariae exposure, animal maintenance, treatment with Niridazole and Oxamniquine, and data collection. No significant differences in mortality patterns (PMT), liver worm burdens (PCT) or surviving worm perfusion data (PMT and life cycle groups) were detected between the two groups. These data, resulting from 10 different experiments are currently being analyzed and evaluated.

RESULTS OF DRUG TESTING:

During FY78 a total of 1,195 selected bottle number drugs were tested for prophylactic (PMT) and/or curative (PCT) activity. Figure 2 depicts the results for the two tests. Of the compounds tested, 290 (44.1 percent) were PMT retests of previously tested drugs and 218 (40.5 percent) were PCT retests. All others were compounds which were tested for the first time. Figure 3 identifies those compounds confirmed as active in one of the two test systems. Primary reference is made only to the bottle code numbers since many (but not all) of the compounds that we receive and test are protected proprietary secrets ("commercially discreet"). We have identified the general class of compound where possible. The identified activity is based upon one or more confirmatory retests; compounds determined to be active on the first test will be confirmed by retest at the same dose by the same route of administration. Only after this confirmation is the drug considered active. Note that five compounds -- BH 08 111, BH 08 157, BH 08 166, BH 08 228 and BH 30 0333 -- have demonstrated both confirmed PMT and PCT activity. It is also worth noting that BH 30 033 has shown unconfirmed activity in a wide range of doses from 10 mgs/kg to 320 mgs/kg. This initial "block" treatment schedule must be repeated for confirmation.

LITERATURE CITED

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A Perfusion Procedure (Perf-O-Suction) for Recovery
of Schistosome Worms. J. Parasit. 47: 366-368, 1961.

FIGURE 1

FY 78 Workload Data for USAMRU-Brasilia Expressed in
Terms of Numbers of Mice Infected

Procedure	Numbers of Mice Infected			
	Control	Test	Total	
	Groups*	Groups*	Number	Percent**
PMT	760	2,735	3,495	27.6
PCT	560	3,350	3,910	30.9
Life Cycle	N/A	N/A	2,956	23.3
Other***	N/A	N/A	2,300	18.2

* = "Control Groups" include those animals designated as uninfected/untreated, infected/untreated, and infected/treated with reference drug(s) control animals. "Test Groups" include only infected animals treated only with Bottle Numbered Test compounds.

** = Percent of total mouse exposure effort.

*** = Includes standardization of the Secondary Curative Test (SCT), comparisons of Walter Reed and Brazilian strains of infection and other schistosomiasis research efforts performed in support of UnB research programs.

FIGURE 2

Drug Test Results for the USAMRU-Brasilia During FY 78

Test System	Total Tests Performed*	Number of Drugs Tested**			
		Confirmed Active	Toxic	Negative/Unconfirmed Active	Total
PMT	547	6	136	396	538
PCT	670	15	32	610	657

* = 1 test = five mice treated with one drug at one dosage by one route of administration. Total number of tests does not include untreated or reference drug control groups.

** = Number of WRAIR/USAMRU-Brasilia bottle number compounds.

FIGURE 3

A List of Bottle Number Drugs Found to be Active Against
Schistosomiasis mansoni by the Mouse Mortality test and
Curative Test Systems During FY78

No.	WRAIR/BRASILIA Bn Number Drug	Prophylactic Test (PMT)*	Curative Test (PCT)*
1.	AD 22 662 (BR 1258)		320 gav
2.	AH 91 461 (BR 1296)		200/320 gav
3.	BC 42 725 (BR 1533)D BH 73 029 (BR 5104)D		40 gav
4.	BD 58 040 (BR 1254) (BR 1025) BE 19 593 (BR 4498)		160 gav
5.	BE 21 922 (BR 1311)		320 SQ & gav
6.	BE 21 931 (BR 1312)	640 SQ FY75+	320 gav
7.	BG 41 577 (BR 1626) (BR 1522) (BR 4502) Nitrovinyl-Furan		40 SQ
8.	BG 52 598 (BR 2079)		80/160 gav
9.	BG 75 064 (BR 2456)		100/200 SQ
10.	BG 81 679 (BR 4405)		100 SQ
11.	BH 08 111 (BR 2889) Organic Tin	640 SQ	100 SQ
12.	BH 08 157 (BR 2893) Organic Tin	640 SQ	100 SQ
13.	BH 08 166 (BR 2894) Organic Tin	640 SQ	100 SQ
14.	BH 08 200 (BR 2897) Organic Tin	640 SQ	
15.	BH 08 228 (BR 2899)	640 SQ	100 SQ
16.	BH 30 033 (BR 3809) Isothiocyanate	1280 SQ	100 SQ (10-320)

* = Confirmed by repeat testing; dosages in mgs/kg;
SQ = Subcutaneous administration; gav = oral
administration by gavage.

+ = Year activity first reported.

D = Discreet Compound

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD DR&E (AR) 636	
3. DATE PREVIOUS SUMMARY ^a	4. KIND OF SUMMARY ^a	5. SUMMARY ACTIVITY ^a	6. WORK SECURITY ^a	7. RESEARCH ^a	8. ORIGIN INSTITUTION ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS ^a	10. LEVEL OF SUMMARY ^a
77 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO. / CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62770A	3M162770A802	00	010			
B. CONTRIBUTING							
*KEYWORDS: CARDS 114F							
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(U) Field studies on Oropouche virus							
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002600 Biology 005900 Environmental Biology							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. FUNDING AGENCY		17. PERFORMANCE METHOD	
73 11		CONT		DA		C. In-House	
18. CONTRACT/GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				PREVIOUS		B. FUNDS (in thousands)	
B. NUMBER: NA				FISCAL YEAR		78 3 100	
C. TYPE: NA				CURRENT		79 3 80	
D. KIND OF AWARD: NA				E. CUM. AMT.			
21. RESPONSIBLE SCS ORGANIZATION				22. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: US Army Medical Research Unit-Belem			
ADDRESS: Washington, D.C. 20012				ADDRESS: APO Miami 34030			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME if U.S. Academic institution)			
NAME: Rapmund, Garrison, COL				NAME: LeDuc, J.W. CPT MSC			
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23. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence not considered.				NAME: Hoch, A.L. CPT MSC			
24. REVISIONS (Precede with Security Classification Code)				25. VIROLOGY; (U) ENTOMOLOGY; (U) EPIDEMIOLOGY;			
(U) MAMMALOLOGY; (U) ORNITHOLOGY; (U) ECOLOGY; (U) BRAZIL							
26. TECHNICAL OBJECTIVE ^a 27. APPROACH 28. PROGRESS (Furnish individual paragraphs identified by number. Precede last of each with Security Classification Code.)							
<p>23. (U) Conduct studies on the ecology of Oropouche (ORO) virus transmission in the Amazon basin in collaboration with the Evandro Chagas Institute. This arbovirus is the most common cause of arbovirus epidemics in the region and commonly results in febrile, debilitating illness. Information on the dynamics of ORO virus transmission in urban and sylvatic environments is of importance to military personnel transiting or stationed in this geographic area.</p> <p>24. (U) Special and routine virological, entomological and epidemiological procedures were employed. Field collected specimens of insects, bird and mammal blood and organs will be processed for virus isolation. Sera will be tested for antibodies to ORO virus to detect areas of recent ORO virus activity.</p> <p>25. (U) 77 10-78 09 A working hypothesis has been formulated in which two cycles are proposed; an overt epidemic cycle and a silent enzootic cycle. In the epidemic cycle <i>Culicoides paraensis</i> is the apparent vector, and man is the vertebrate amplifying host. In the enzootic cycle the vector is unknown, and the vertebrate hosts are most likely birds or primates. The epidemic cycle occurs in urban areas, the enzootic cycle in sylvatic habitats. Investigations of the epidemic vector <i>Culicoides paraensis</i> have shown: (A) a diurnal activity pattern; (B) anthropophilic feeding preferences; (C) strong endophilic tendencies; (D) a widespread geographic distribution; (E) a seasonal distribution which indicates activity throughout the year, but members increase during the rainy season and diminish during the dry season; and (F) rotting banana stalks are the preferred breeding site, which indicates a strong association with man and a potential control method through modification of current social practices; (G) ability to experimentally transmit ORO virus. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 October 1977-30 September 1978.</p>							

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1143

PROJECT 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 010 FIELD STUDIES ON GROPOUCHE VIRUS

Investigators

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INTRODUCTION AND SUMMARY OF ACCOMPLISHMENTS.

The USAMRU-Belém is located in Belém, Brazil and works in close conjunction with and has laboratory space at the Institute Evandro Chagas, a research center administered by the Brazilian Public Health Service (FSESP). The sole area of investigation for the USAMRU-Belém is the ecology of arbovirus diseases. Current efforts attempt to elucidate the endemic and epidemic cycles of Oropouche (ORO) virus. This virus causes disease in man generally of about 7 days duration, with some patients severely ill, occasionally to the point of prostration. This disease has been reported to cause large scale epidemics in urban areas of northern Brazil.

Accomplishments included in this report with regard to ORO virus have been divided into two major categories; studies on the epidemic cycle, and studies on the endemic cycle. Under the category studies on the epidemic cycle, conclusive data is presented which establishes the midge, Culicoides paraensis, as the primary epidemic vector of ORO virus. Subsequent sections present results of investigations of the basic biology of this midge, all of which will be of immense value should effective control measures be desired, such as would be necessary to combat an epidemic of ORO virus disease.

A second section of studies on the epidemic cycle of ORO virus deals with man as the principal vertebrate host in the epidemic cycle. Here evidence is presented which indicates that, when infected, man circulates ORO virus in sufficient titer to infect feeding C. paraensis.

Finally, results of an epidemic of ORO virus disease which occurred in and around Tomé Acú, Pará, Brazil are reported. These results provide striking verification of the theories proposed in the previous two sections. Clearly, C. paraensis is the predominant vector in this outbreak, and in all likelihood man is the only amplifying host.

Investigations of the endemic cycle of ORO virus are also reported. Three separate sites were sampled during 1977-1978, Curuá-Una, Mojui dos Campos and Cachoeira Porteira, all located in Pará, Brazil. Vertebrates were sampled from all 3 localities, and insects were sampled at 2 of the 3 sites. Oropouche virus was not found among any insects tested; however, results of the serological surveys indicate that primates and certain groups of birds had been previously exposed the virus.

In early 1978 the USAMRU was invited to participate in investigations of an epidemic of unknown etiology which was in progress in

Belterra, Pará, Brazil. It was soon determined that Yellow Fever (YF) and Mayaro (MAY) viruses were both responsible for this epidemic. The occurrence of epidemic MAY virus provided a unique opportunity to study a virus known to cause disease in man in the Amazon Basin, but previously reported only incidentally.

Studies of MAY virus are organized in the same format as those for ORO virus; studies on the epidemic cycle, and studies on the endemic cycle. Studies on the epidemic cycle describe in detail the epidemic of Belterra.

Following a brief synopsis of the literature pertaining to MAY virus and a detailed description of Belterra, a summary of the clinical manifestations of MAY virus infection in man is presented. These results indicate that infection with this virus results in severe, debilitating disease of approximately one week's duration. No deaths were attributed to infection with MAY virus. It was especially fortunate that a complete census of Belterra had just been completed in December, 1977, consequently accurate denominator information was available for accurate estimates of attack rates. A clinically apparent attack rate of between 89 and 96% was estimated for those infected among all age groups in Belterra, and from 94 to 100% among that segment of the population over 10 years old. Approximately 20% of the 4000 residents of Belterra were infected during this epidemic.

Subsequent sections deal with vectors and vertebrates involved in the epidemic cycle of MAY virus. Entomological investigations indicate that the principal, if not only vector of MAY virus in this epidemic was Haemagogus janthinomys. Noteworthy, this was also the only epidemic vector of YF virus recognized as well. Studies of the vertebrate population indicate that a small marmoset, Callithrix argentata, was most likely the primary vertebrate amplifying host of MAY virus in the outbreak. This was supported by the isolation of MAY virus from a feral marmoset, and subsequent laboratory demonstration of a considerable viremia produced following experimental infection with Mayaro virus.

Studies on the endemic cycle of MAY virus, also conducted at Curua-Una and Cachoeira Porteira, failed to isolate the virus. Serological results of the vertebrates sampled indicate that primates have a high antibody prevalence rate to MAY virus, but few other groups of vertebrates were found with antibody.

The epidemic of MAY virus investigated at Belterra indicates that the epidemiology of this virus is different from that proposed for ORO virus. Apparently MAY virus is restricted to a sylvatic cycle similar to jungle YF virus. An urban vector capable of

maintaining massive outbreaks as occurs with C. paraensis and ORO virus is apparently lacking. This does not, however, lessen the significance of MAY virus to military medicine. The population studied at Belterra shares many of the characteristics of a military cantonment area, and the fact that approximately 20% of this population was infected, including representatives of all age groups and both sexes, indicates that disease transmission was widespread throughout the area. An epidemic of this magnitude could have a significant impact on military operations in the Amazon Basin.

I. ECOLOGY OF OROPOUCHE VIRUS

A. Studies on the Epidemic Cycle

1. Vector biology

a. Laboratory transmission studies

OBJECTIVE: Objective of the Oropouche virus transmission studies was to further substantiate preliminary results with Culicoides paraensis (Goeldi) and Culex pipiens quinquefasciatus (Say).

BACKGROUND: Oropouche (ORO) virus was first discovered in Brazil in 1960, and subsequently it has become recognized as an important public health problem due to its occurrence in several urban epidemics in the Amazon Basin. Epidemiological and entomological investigations conducted during these epidemics have strongly indicated that ORO virus was vector-borne. Culex pipiens quinquefasciatus and Culicoides paraensis were considered to be the most probable vectors of the virus due to their common occurrence and superior numerical abundance in all epidemics investigated.

In order to determine the vector potential of Cx. p. quinquefasciatus and C. paraensis, laboratory transmission studies were conducted under controlled conditions using hamsters as the model host. Biological transmission of ORO virus was demonstrated for both species during the preliminary transmission trials; however, C. paraensis appeared to be the better vector of the two. The extrinsic incubation period for ORO virus in C. paraensis appears to be 5 to 7 days, but was not determined for Cx. p. quinquefasciatus. The threshold viremia level required to infect feeding C. paraensis and Cx. p. quinquefasciatus was also not established.

DESCRIPTION: Culicoides used in the transmission studies were collected at a field station where the midge populations remains active throughout the year. Oropouche virus is not known to exist within this collecting area. Culex p. quinquefasciatus used in the test were 1st generation mosquitoes reared from wild caught populations obtained from the Belém urban area.

Virus strains: Oropouche virus, strain Br An 19991, was used to inoculate the experimental animals. Young hamsters (21-23 days of age) were used as the donor and recipient vertebrate hosts in all transmission experiments.

Virus Titration: Hamsters were inoculated intracerebrally with 0.1 ml of undiluted hamster serum containing ORO virus. Twenty to 24 hrs. following inoculation, 0.1 ml of blood was obtained from the hamster by cardiac puncture and immediately added to 0.9 ml of

phosphate buffered saline (PBS) containing 0.4% bovine albumin. This 1:10 dilution was further diluted and titrated in Vero cells or suckling mice to determine the titer of ORO virus in the donor hamster. After bleeding, the hamsters were immediately exposed to insects included in the transmission tests.

Virus Isolation and Identification: Individual insects were homogenized and suspended in PBS containing bovine albumin and antibiotics. A 1.5 ml aliquot of diluent was used for Culex mosquitoes and 1.0 ml was used for Culicoides.

After centrifugation of the insect homogenate at 1500 RPM, the supernatant fluid was aspirated and 0.1 ml was inoculated into each of 3 tubes of Vero cells. The tubes were observed every 2 days to detect viral cytopathic effect (CPE). Tubes demonstrating a 3 to 4 + CPE were harvested and frozen at -60°C. To identify the virus, a 1:100 dilution of the infected fluid was mixed with equal amounts of ORO virus hyperimmune mouse ascitic fluid, incubated for 1 hour at 37°C, then assayed for infectivity. These tests were performed in microtiter plates to which Vero cells were added after incubation of the virus and virus-serum mixtures. The test control series consisted of infected fluids without additions of the ORO virus hyperimmune mouse ascitic fluids. The tests were routinely read 3-4 days post-inoculation, or when the virus controls showed a 3 - 4 + CPE.

PROGRESS: Tables 1 and 2 present the results of attempts to transmit ORO virus to susceptible hamsters by Cx. p. quinquefasciatus which fed on infected hamsters circulating ORO virus. As shown in Table 1, Cx. p. quinquefasciatus were fed on a hamster with a viremia of $10^{4.6}$ SMLD₅₀/0.02 ml of ORO virus. Repeat feeding of "infected" Culex on susceptible hamsters were conducted on days 7, 14, 21 post-exposure. After the final feeding, hamsters were observed for an additional 3 week period for clinical signs of ORO virus. If clinical signs of ORO virus were not observed, then the hamster was sacrificed and serologically tested for antibody to ORO virus. As the data indicates, none of the 30 susceptible hamster in this experiment showed clinical signs of ORO virus, and all serological tests were negative for antibody to ORO virus.

Table 2 summarizes the results seen where 4 hamsters with viremias of 10^5 , 10^5 , $10^{5.4}$ and $10^{5.3}$ SMLD₅₀/0.02 ml which were fed on by 4 separate lots totalling 210 Cx. p. quinquefasciatus. Susceptible hamsters were subsequently exposed to each lot in the same manner as described for hamsters in Table 1. None of the 60 susceptible hamsters used in the these tests showed clinical signs of ORO virus infection or exhibited antibody to ORO virus.

Table 3 presents the results of attempts to transmit ORO virus to susceptible hamsters by C. paraensis which fed on infected hamsters

circulating $106.6\text{SMLD}_{50}/0.02\text{ ml}$ of ORO virus, and results of virus isolation attempts from midges following feeding on susceptible hamsters. These results show that 4 of 5 (80%) Culicoides assayed following feeding on susceptible hamsters harbored ORO virus; however, only 1 (25%) of the 4 infected Culicoides actually transmitted ORO virus to a susceptible hamster.

Culicoides which did not feed on the susceptible hamsters on the 7th day of exposure were sacrificed and individually tested for the presence of ORO virus. From twelve Culicoides tested, 3 (25%) ORO virus isolations were made.

Table 4 presents the results of attempts to transmit ORO virus to susceptible hamsters by C. paraensis fed on infected hamsters circulating $105.0\text{SMLD}_{50}/0.02\text{ ml}$ of ORO virus, and virus isolations from midges following feeding on susceptible hamsters. These findings show that ORO virus was transmitted to 5 (83%) of the 6 susceptible hamsters exposed to "infected" Culicoides. Each infection resulted in the death of the hamster. Seven (54%) of the 13 Culicoides which fed on the hamsters on day 7 were confirmed to be infected with ORO virus.

Culicoides which did not feed on the susceptible hamsters on the 7th day of exposure were sacrificed and individually tested for the presence of ORO virus. Twelve Culicoides were tested and 5 isolates (42%) of ORO virus were made. One of these infected Culicoides probably fed undetected on the positive hamster whose associated Culicoides was negative in Table 4.

COMMENTS: In an effort to summarize the laboratory transmission results of ORO virus, a brief discussion of the preliminary results presented in the annual report year of 1976-77 will be included in the present comment section.

Initial biological transmission of ORO virus by Cx. p. quinquefasciatus was demonstrated on 2 occasions during the 1976-77 investigations. The 1st transmission of ORO virus to a susceptible hamster was achieved when a test lot of Culex were fed on a hamster circulating $108.2\text{SMLD}_{50}/0.02\text{ ml}$ of virus. The ORO virus transmission occurred on the 8th day when the experimental hamster was fed upon by 21 "infected" mosquitoes. Oropouche virus transmission to this hamster was confirmed by serological test. Transmission of ORO virus was again demonstrated in another experiment in which a Culex lot had fed on a hamster circulating $107.8\text{SMLD}_{50}/0.02\text{ ml}$ of virus. Oropouche virus was isolated from a susceptible hamster fed upon by a group of 33 Culex, 21 days post exposure. Three later transmission trials were attempted with Culex feeding on viremic hamsters circulating 107.0 , 108.2 , and $108.0\text{SMLD}_{50}/0.02\text{ ml}$ of virus; however, all the 15 susceptible hamsters exposed to refeeding by "infected" Culex were not infected.

Based on these results, it was concluded that Cx. p. quinquefasciatus demonstrated a high infectivity threshold and was apparently an inefficient vector of ORO virus.

Due to the extended colonization of the test Culex material within the laboratory environment, it was felt that the vector competence of the laboratory colony may have been modified, and was no longer representative of the wild urban Culex population. Therefore, the transmission experiments were modified and performed with 1st generation Culex adults obtained from urban areas of Belém. These results are presented in Tables 1 and 2. The data in these tables shows that a composite total of 283 Culex were fed upon 90 susceptible hamsters. Serological tests of all exposed hamsters were negative for antibody to ORO virus. The Culex pools which fed on these hamsters are being tested for the presence of ORO virus.

A review of ORO virus transmission experiments with C. paraensis presented earlier shows that ORO virus was transmitted to 8 susceptible hamsters by Culicoides which had fed previously on viremic hamsters which titrated $10^{6.0}$, $10^{6.5}$, $10^{7.2}$ and $10^{8.2}$ SMLD₅₀/0.02 ml of ORO virus. The earliest recorded successful transmission was on day 4 post-exposure, with a majority of the successful transmissions occurring between days 5 and 8. Of the 8 hamsters infected, 4 (50%) were fed upon by 2 or fewer Culicoides. In addition, ORO virus was isolated from 13% (28/208) of the individually tested Culicoides which were sampled from both fed and unfed individuals. The 13% positive for ORO virus is thus not an infectivity rate, since many of those tested had not received an initial infective blood meal.

In the transmission trials reported here the methods of separating the "infected" midge population from the "non-infected" population were modified and greater standardization in the population of "infected" Culicoides was obtained. Restrained viremic hamsters were exposed to C. paraensis for a period of approximately 45 minutes. Following the exposure period, midges were subjected to cool temperatures to reduce their movement. With the use of a stereomicroscope, blood engorged midges could be separated from the non-engorged. Therefore, only blood engorged midges were used in the subsequent transmission trials.

Results presented in Table 3 indicate that ORO virus was transmitted to susceptible hamsters by one (25%) of 4 infected Culicoides which fed singly. In a second experiment presented in Table 4, where as many as 3 individual Culicoides were allowed to feed on a susceptible hamster, ORO virus was transmitted by 4 (80%) of 5 infected groups of Culicoides. Among those groups tested, 3 (75%) of the 4 positive transmissions were accomplished by single infected Culicoides. Taken together, these results indicate a

range of transmission rates among infected Culicoides from 25 to 80%. These results suggest that Culicoides are fairly efficient vectors, and are certainly better vectors than Cx. p. quinquefasciatus.

It is difficult to explain why the transmission rates were higher for those Culicoides which fed on the lower titered infectious blood meals. One possible explanation is a difference in age among individuals tested in these experiments. Since all Culicoides tested were wild caught as adults, it is impossible to know the age of individuals tested. A second possibility is that these results reflect the normal range of susceptibility among the wild population, and that too few replicates have been conducted to be more specific.

In summary, laboratory transmission of ORO virus has been demonstrated for both Cx. p. quinquefasciatus and C. paraensis. These species represent the dominant nocturnal endophilic and the dominant diurnal endo/exophilic species respectively, and both have been reported to feed on man in all epidemics of ORO virus. Based on the experimental transmission studies reported here, it appears that C. paraensis is the predominant epidemic vector of ORO virus in the urban environment. Subsequent sections of this report attempt to define the basic biology of this vector species.

TABLE 1. Summary of attempts to transmit Oropouche virus to susceptible hamsters by Culex pipiens quinquefasciatus fed on infected hamsters circulating $10^{4.6}$ SMLD₅₀/0.02 ml of Oropouche virus.

Days post-infectious blood meal	Number of susceptible hamsters	No. Mosquitoes Fed	Transmission* results
7	7	7	Neg.
14	9	9	Neg.
15	4	33	Neg.
21	10	24	Neg.
TOTAL	30	73	

* Serological tests were performed 21 day after last exposure to experimental insect population. Serological confirmation was determined by Neutralization test.

TABLE 2. Summary of attempts to transmit Oropouche virus to susceptible hamsters by Culex pipiens quinquefasciatus fed on infected hamsters circulating 105 to 5.4 SMLD₅₀/0.02 ml of Oropouche virus.

Days post-infectious blood meal	Number of susceptible hamsters	No. mosquitoes fed	Transmission* results
7	5	17	Neg.
8	15	61	Neg.
14	20	35	Neg.
21	20	97	Neg.
TOTAL	60	210	

* Serological tests were determined by Neutralization test 21 days after last exposure to experimental insect population.

TABLE 3. Summary of results of attempts to transmit Oropouche virus to susceptible hamsters by *Culicoides paraensis* fed on infected hamsters circulating $106.6 \text{ SMLD}_{50}/0.02 \text{ ml}$ of Oropouche virus, and associated virus isolations from midges following feeding on susceptible hamsters.

Days post-infectious blood meal	No. fed/hamster	Transmission results	Virus isolated from midges following feeding
7	1	Neg.	Pos.**
7	1	Neg.	Neg.
7	1	Neg.	Pos.
7	1	Neg.	Pos.
7	1	Pos.*	Pos.

* All pos. results confirmed by Complement Fixation test.

** All pos. results confirmed by Neutralization test.

TABLE 4. Summary of results of attempts to transmit Oropouche virus to susceptible hamsters by *Culicoides paraensis* fed on infected hamsters circulating 105.05MLD₅₀/0.02 ml of Oropouche virus, and associated virus isolations from midges following feeding on susceptible hamsters.

Days post-infectious blood meal	No. fed/hamster	Transmission results	Virus isolated from midges following feeding
7	2	Pos.*	2 Pos.**
7	3	Pos.	1 Pos.-2 Neg.
7	2	Neg.	2 Pos.
7	1	Pos.	1 Neg.+
7	3	Pos.	1 Pos.-2 Neg.
7	2	Pos.	1 Pos.-1 Neg.

* All pos. results confirmed by Complement Fixation test.

** All pos. results confirmed by Neutralization test.

+ Likely that this hamster was fed upon by an undetected infected *C. paraensis*

b. Urban seasonal abundance of Culicoides paraensis

OBJECTIVES: The objectives of this section are to:

- a. determine the seasonal activity pattern for Culicoides paraensis within the urban environs of Belém.
- b. demonstrate a relationship between seasonal rainfall and midge population activity.

BACKGROUND: Epidemics of Oropouche (ORO) virus have been documented in several urban areas within the state of Pará, Brazil since 1961: Belém, Bragança, Itupiranga, Mojui dos Campos, Santarém, Belterra and Tomé Açú. In all epidemics investigated, two insect species have been the most common human feeders: Culex pipiens quinquefasciatus nocturnally and Culicoides paraensis diurnally. Laboratory transmission studies of ORO virus with both species have previously demonstrated that C. paraensis is the more efficient of the two species, and these results have formed the basis for our working hypothesis that this species is the principal epidemic vector of ORO virus. In this and subsequent sections, discussions of the basic biology of C. paraensis are presented. This information is essential in the development of effective disease prevention and/or control strategies.

DESCRIPTION: Seasonal population studies for C. paraensis were conducted in a peridomiliary environment near (10-20 meters) houses in 2 separate city zones of Belém, Pará, Brazil. Landing captures were performed by a team of two collectors who captured Culicoides attempting to feed on the exposed portion of the lower leg (knee to ankle). Captures were conducted for 4 consecutive 30 min. intervals from 14:00-16:00 hrs. at each collection site for 2 consecutive days on alternate weeks. The study was initiated in July, 1977 and was continued through July 1978. Rainfall data was obtained from an agriculture experimental research station (CEPLAC) located on the periphery of Belém, Pará, Brazil.

PROGRESS: Figure 1 presents the mean hourly numbers of C. paraensis collected per month, and total monthly rainfall. It is apparent from the rainfall data that the municipality of Belém receives a voluminous quantity of rainfall annually. In only one month (November) was the total rainfall measured below 75 mm. It is also apparent that the monthly rainfall shows a definite seasonal change, with those months of December to March recording

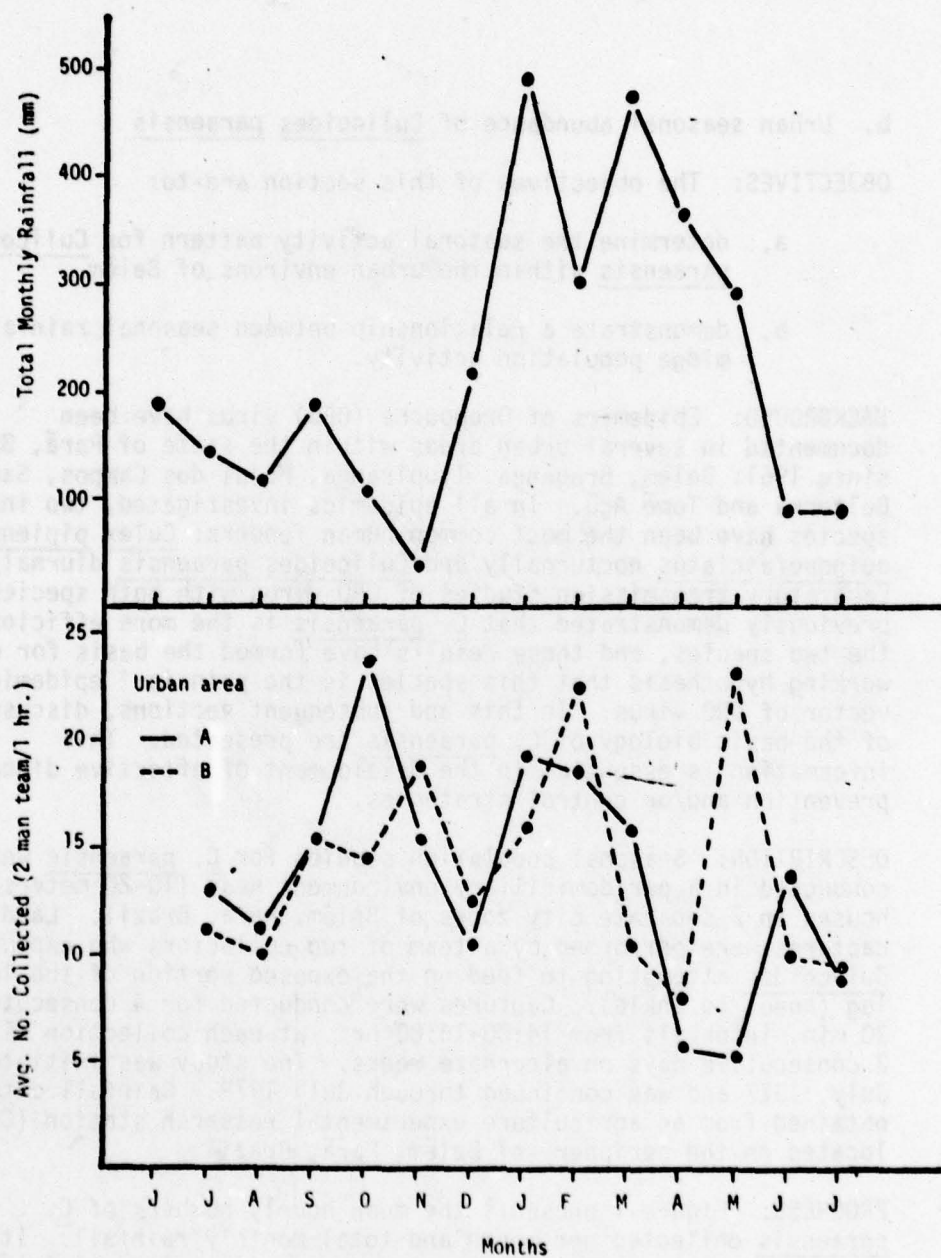


FIG. 1 . Peridomestic population patterns of *Culicoides paraensis* (Goeldi) collected at two separate urban locations in the city of Belém, Pará, Brazil, 1978

the greatest quantity.

The population data for C. paraensis demonstrates that Culicoides were active in the urban areas through out the year, exhibiting a trimodal increase in Culicoides numbers for the 13 month observation period. The collection results show that the midge population fluctuated from 7 to 25 Culicoides for the mean hourly captures per month with peaks of activity occurring in the months of: September-November, January-February, and May-June.

COMMENTS: The collection data from the two areas of Belém demonstrates a corresponding rise and fall in the midge population. If one assumes that the population patterns of Culicoides are directly related to environmental factors, then one would conclude that the environmental conditions for the two areas are relatively similar. In addition, one would expect to observe a correlation between the environmental parameters and midge population patterns. When comparing the population and rainfall pattern, one will note a general synchronous rise and fall of the two parameters. Thus, it was assumed that the Culicoides population is markedly influenced by the quantity of seasonal rainfall.

The influence of rainfall on midge population was expected since C. paraensis breeds in aquatic to semiaquatic habitats. It will be demonstrated in a subsequent section that decomposing banana tree vegetative matter is a major breeding habitat for C. paraensis within an urban environment. The decaying vegetative substrate provides the necessary wet conditions and food substances required for midge larval development. Since immature stages of Culicoides require a semiaquatic environment to survive and/or develop, it then becomes apparent that a critical amount of moisture must be maintained/or received to create favorable habitat conditions. However, due to the good water retention characteristics of the banana substrate, this habitat will retain its favorable breeding conditions for short intervals of reduced rainfall. Nevertheless, the natural water losses must be renewed periodically by rainfall to maintain a viable breeding site. The question then arises, how much rainfall is necessary to provide the minimum critical moisture conditions for the breeding habitats.

It is also worthy to note that a lag time of approximately one month exists between the initial increase in rainfall and a corresponding increase in the midge population. This will be noted when comparing the increase in rainfall in September followed by a population increase for Culicoides in October and November (Figure 1). This pattern is observed again when the rainfall increased in December with a subsequent increase in midge numbers in January and February. This lag time can be explained in part by the slow developmental period observed for Culicoides larvae to reach the adult phase. Controlled laboratory studies indicate that the

developmental cycle (egg to adult) for Culicoides is approximately one month.

It is difficult to directly associate the quantity of rainfall with the variations noted in the population of Culicoides. For example, during the rainy season (January to May) it was observed that the midge population fluctuated markedly during a part of the season which was receiving more than 300 mm of rainfall per month. It would be reasonable to assume that sufficient rainfall was being received to provide favorable habitat conditions and to maintain a high population of adult Culicoides. Nevertheless, a marked reduction in the population numbers was noted. At this time, data is not available to explain the noted population reduction occurring during the rainy season. However, it is conceivable that heavy rainfall may result in adult midge mortality caused by drowning.

In summary, the population activity for C. paraensis was found to be continuous throughout the year in the urban study areas of Belém. Collection data tends to support the association of Culicoides activity with seasonal rainfall.

c. Daily activity patterns of Culicoides paraensis

OBJECTIVE: The objectives of the following investigations are to quantify the daily activity pattern of Culicoides paraensis in 3 principal domestic environs, and to monitor designated environmental parameters in each environ.

BACKGROUND: Epidemiological and entomological observations of hematophagous insects effecting man in his urban environment indicate that C. paraensis is frequently the most common and dominate species. Information collected previously concerning the daily activity of this species indicates that they are strictly diurnal blood feeders, with apparent regulated behavioral activity patterns. It has also been noted that the biting pressure observed within a particular domestic environment is significantly variable. Therefore, it was hoped that observations of certain environmental factors within a particular domestic setting might help to explain the differences observed in C. paraensis activity pattern.

DESCRIPTION: The domestic environment evaluated in this investigation is located on the property of an agriculture research station near experimental cacao plots. This location was chosen due to the high population of C. paraensis that existed in the area and the absence of adjacent housing units. Known breeding sites for C. paraensis within this area were those of decaying vegetative matter of banana trees and cacao pods. Fortunately the diurnal midge population of this area was essentially a monoculture of C. paraensis.

The house used in the study was a simple 5 room house constructed of stucco walls and a tile roof. During the day, as is custom in tropical regions, the doors and windows were left open for natural ventilation. The family unit consisted of 8 members. Domestic animals consisted of a dog and a few caged chickens. No large domestic animals such as cattle, sheep or pigs were known to exist within a one kilometer radius of the study site; therefore, man was the dominate host for blood-feeding midges.

A few shade trees of various sizes and heights were maintained in the yard. A group of larger trees flanked one side of the house at a distance of 7 to 10 meters. These trees provided a continuously shaded area throughout the day.

The domestic environs evaluated in this investigation were intradomiciliary and peridomiciliary. Two peridomiciliary sites were located at approximately 10 to 15 meters from the house. One site was under a group of shade trees while the other site was in the open a few meters (10 meters ca.) from both the shade trees and house.

A team of two collectors were stationed at each of the 3 collecting sites, and each team performed biting collections for a period of 45 minutes per hour. Each collecting team was rotated hourly among the 3 collecting sites to prevent differences caused by collector efficiency. Continuous hourly captures were conducted between 06:00 to 18:45 hours. Observations were made 3 times a week for approximately one month's duration. Collecting containers were gathered periodically to enumerate the number of Culicoides captured in each area and to determine the species present. Hydrothermographs were used to monitor temperature and relative humidity.

PROGRESS: A comparative summary of the results obtained during this study is presented in Figure 2. With little variation, midge biting activity was noted to begin at approximately 06:00-06:15 hrs each day during the month of observation. The activity data show that there was an early morning peak in biting activity in the house, followed by a steady decline in activity until 10:00 hrs.; subsequently, a moderate midday increase in activity was observed. The early morning peak for the exposed peridomiciliary site was followed by a decline in activity until 16:00 hrs in the evening. A marked early morning biting peak was not observed for the shaded peridomiciliary site; however, a general increase in biting activity was recorded until midday, after which a marked decrease in biting activity occurred until 16:00 hrs. At 16:00 hrs all sites showed a synchronous increase in biting activity. The late evening activity period lasts for approximately one hour before a sharp decline in numbers is noted. Shortly after 18:30, all biting activity for C. paraensis stops.

The temperature data shows that the early morning (06:00-09:00 hrs) temperatures and late evening temperatures (17:00-18:00 hrs) for the three sites vary only a few degrees of each other, while the mid-afternoon temperatures (13:00-16:00 hrs) show the largest range of temperature readings. The site recording the highest temperatures was the exposed site, while the inhouse readings were intermediate between the two peridomiciliary temperature readings.

Relative humidity readings were near 100% at the beginning of the collections and fell to below 90% between the hours 08:00 and 17:00 hours. The exposed peridomiciliary site showed the lowest level of humidity

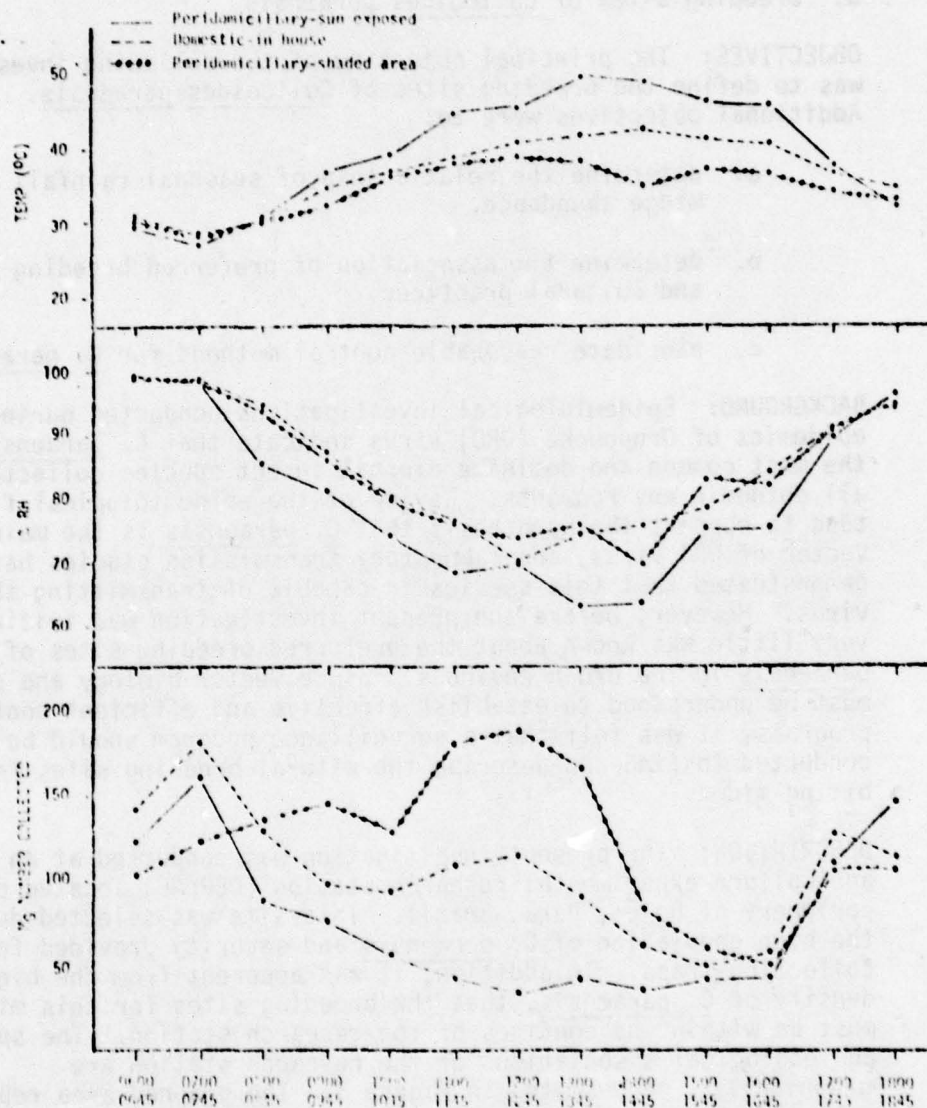


FIG. 2. Diurnal activity pattern of *Calliphora vicina* (Gould) determined in 3 different environmental settings: domestic (1) in house; Peridomestic (2) area; shaded - (3) area exposed to the sun, Pelion, Para, Brazil, 1978.

d. Breeding sites of Culicoides paraensis

OBJECTIVES: The principal objective of the following investigation was to define the breeding sites of Culicoides paraensis. Additional objectives were to:

- a. determine the relationship of seasonal rainfall and midge abundance.
- b. determine the association of preferred breeding sites and cultural practices.
- c. elucidate reasonable control methods for C. paraensis.

BACKGROUND: Epidemiological investigations conducted during epidemics of Oropouche (ORO) virus indicate that C. paraensis was the most common and dominate diurnal insect species collected in all epidemic environments. Review of the epidemiological findings tend to support the hypothesis that C. paraensis is the main urban vector of ORO virus, and laboratory transmission studies have demonstrated that this species is capable of transmitting this virus. However, before the present investigation was initiated, very little was known about the preferred breeding sites of C. paraensis in the urban environs. Since vector biology and ecology must be understood to establish effective and efficient control programs, it was felt that a surveillance program should be conducted to find and describe the natural breeding sites for this biting midge.

DESCRIPTION: The present investigation was conducted at an agriculture experimental research station (CEPLAC) located on the periphery of Belém, Pará, Brazil. This site was selected due to the high population of C. paraensis and security provided for collecting traps. In addition, it was apparent from the high density of C. paraensis, that the breeding sites for this midge must be within the confines of the research station. The spatial and ecological associations of the research station are graphically represented in Figure 3. The graphed area represents only a small portion of the actual area involved in cacao production; however, it was not felt that those areas outside the immediate collecting sites were influencing the midge population in the study area.

Ecologically, the study site would be described as an mixed cacao

and banana tree plantation. The planting of banana trees in cacao plots is a common cultural practice and is done so that the banana trees will provide protective shade for young cacao trees. Adjacent areas of secondary forest and lowland marsh bordered the area of investigation.

Emergent traps were used to survey different ecological habitats for breeding of *C. paraensis*. The type of emergent trap utilized was dependent upon the ecological habitats being evaluated. Description of the habitat types and emergent trap designs were included in the 1976-77 Annual Report. Therefore, only the breeding sites will be briefly described in this section.

Since the breeding habitats in the study environment were unknown, it was felt that several habitats would have to be investigated to determine the preferred breeding sites. The habitats evaluated were: (1) processed cacao hulls discarded in open piles to decompose; (2) copious layers of cacao leaf litter lying below the cacao trees; (3) cut banana stumps, that portion remaining after the main trunk had been removed; (4) decomposing banana stalks laying on the ground within the cacao and banana plots, and (5) the marsh area composed of a rich organic composite of decaying vegetative matter and stagnant water.

Insect collecting containers employed with each emergent trap were constructed with an alcohol reservoir for preserving the small bodied *Culicoides*. These reservoir cups were routinely collected to process the captured specimens. Since the purpose of the study was to define the breeding sites of *C. paraensis*, this species was enumerated, while counting and volumetric techniques were used to quantify the other midge species. Emergent traps utilized in the different habitats were relocated periodically to allow continuous observations of the particular study habitats.

Monthly rainfall data was collected within the CEPLAC research station.

The adult population of *C. paraensis* was also monitored daily at the research station by a team of two collectors making biting counts on the lower exposed portion of the legs for two sequential 15 minute biting captures.

PROGRESS: Data obtained from emergent traps indicate that *C. paraensis* were breeding in three of the five habitat types investigated (Table 5). Two of these habitat types were associated with decaying banana vegetative substrate and the third breeding site was decaying cacao hulls. Of the two banana tree substrate habitats, banana stalks were shown to be the preferred breeding site by greater numbers of emerging midges.

The third breeding site for C. paraensis was shown to be decomposing cacao hulls. Emergent trap data for the cacao leaf litter and marshland habitats were negative for C. paraensis and thusly, collections in these habitats were discontinued in February and March, respectively. However, other species of Culicoides were collected in the marshland habitat.

Table 6 presents the quantitative composition of the Culicoides fauna in the two principal breeding habitats represented. Culicoides paraensis is the principal species of Culicoides breeding in the decaying banana stalk habitat; however, in the cacao habitat contributed considerably less to the total Culicoides species present. Some of the species associated with the cacao and banana habitats are also presented in Table 6.

Figure 4 presents the total monthly rainfall and the number of C. paraensis recorded per trap-day collected from the cacao hull and banana stalk habitats. It is apparent from the data available that there is considerable variation in emergent pattern between the two breeding habitats. The emergent population from the cacao habitats appears to be more directly related to the rainfall pattern than the results obtained from the banana stalk breeding site.

Figure 5 illustrates the monthly population activity pattern for C. paraensis within the study area. It would appear from these data that the breeding pattern is associated with the quantity of rainfall. Moreover, the data tend to demonstrate that a high quantity of rainfall is required to sustain a larger population of C. paraensis at a relatively stable population level.

Figure 6 shows the effects of a dust insecticide (BHC 1.5%) on the population of C. paraensis when the experimental cacao trees were being treated for insect pests. The population showed a temporary decline following the application of insecticide; however, it is noted that by the 3rd and 4th day post-treatment, the population had returned to numbers equivalent to the pre-treatment levels.

COMMENTS: Data obtained in this breeding site surveillance program shows that rotting banana vegetative materials and decomposing cacao hulls are the preferred breeding habitats in the study area.

At first it would appear that the banana stumps and stalks should be considered the same habitat; however, after noting the quantity of water associated with the two banana substrates, it was felt that a habitat distinction was warranted. The upright banana stumps function as an artificial reservoir for rain water, thus creating an aquatic environment, while the decaying banana stalk on the ground creates a semiaquatic environment due to its moisture retention properties. The separation of these two habitats is also supported by the emergence data presented in Table 5. It is

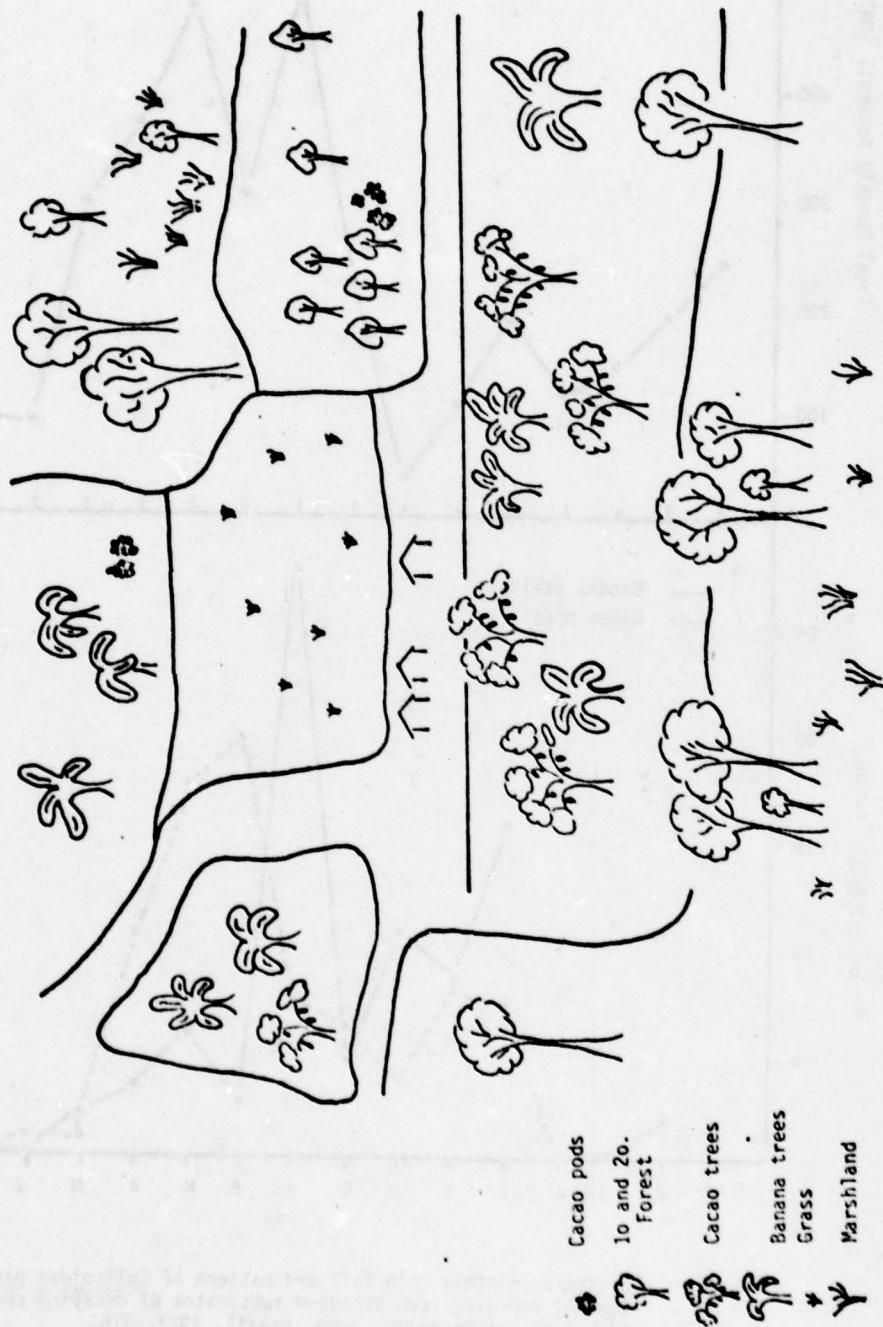


FIGURE 3. Spatial and ecological association of breeding site habitats at a cacao research station Belém, Pará, Brazil, 1977-78.

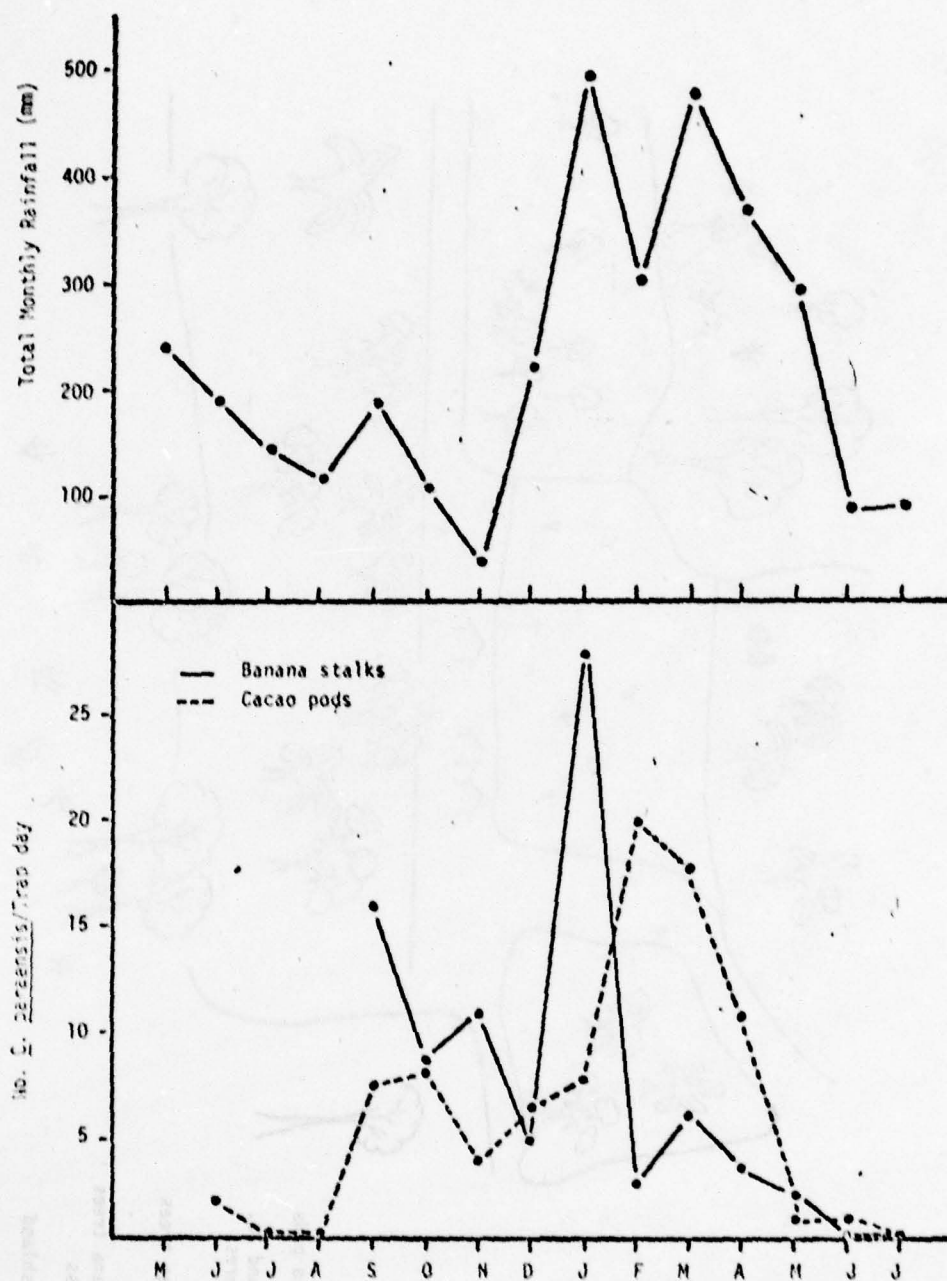


FIG. 4 . Cumulative monthly rain fall and pattern of *Culicoides paraensis* (Goeldi) emerging from breeding substrates of decaying cocoa pods and banana stalks, Belém, Pará, Brazil, 1977-1978.

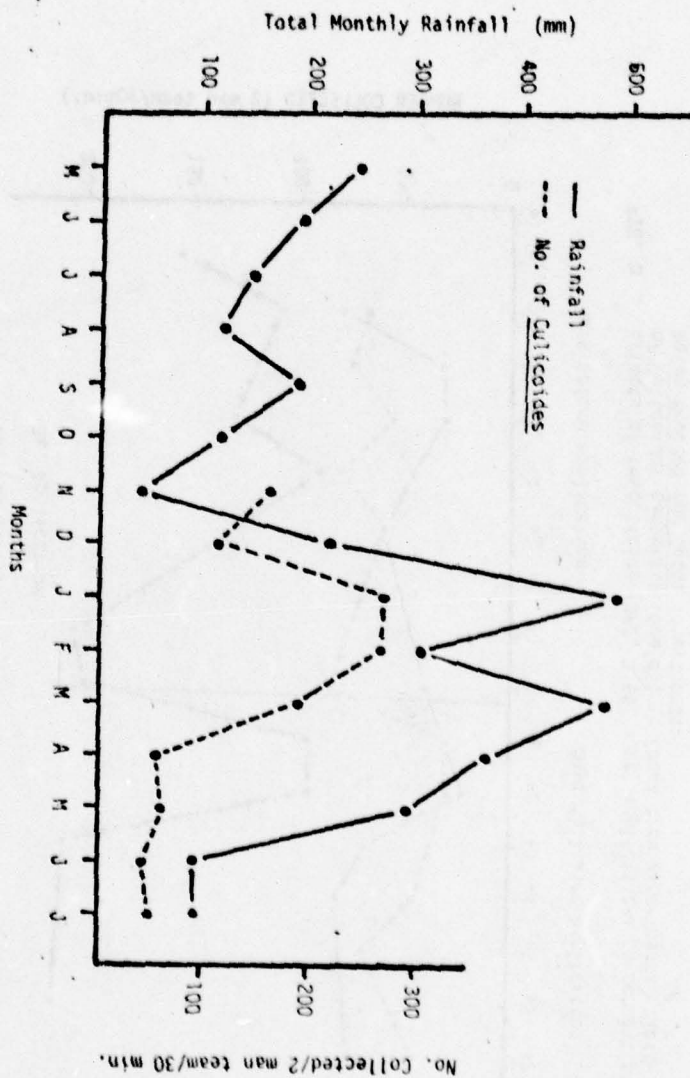


FIG. 5. Comparison of monthly rainfall and population patterns of *Culicoides paraensis* (Goeldi) collected near their breeding habitats in a Cocoa experiment research station, Para, Brazil, 1978

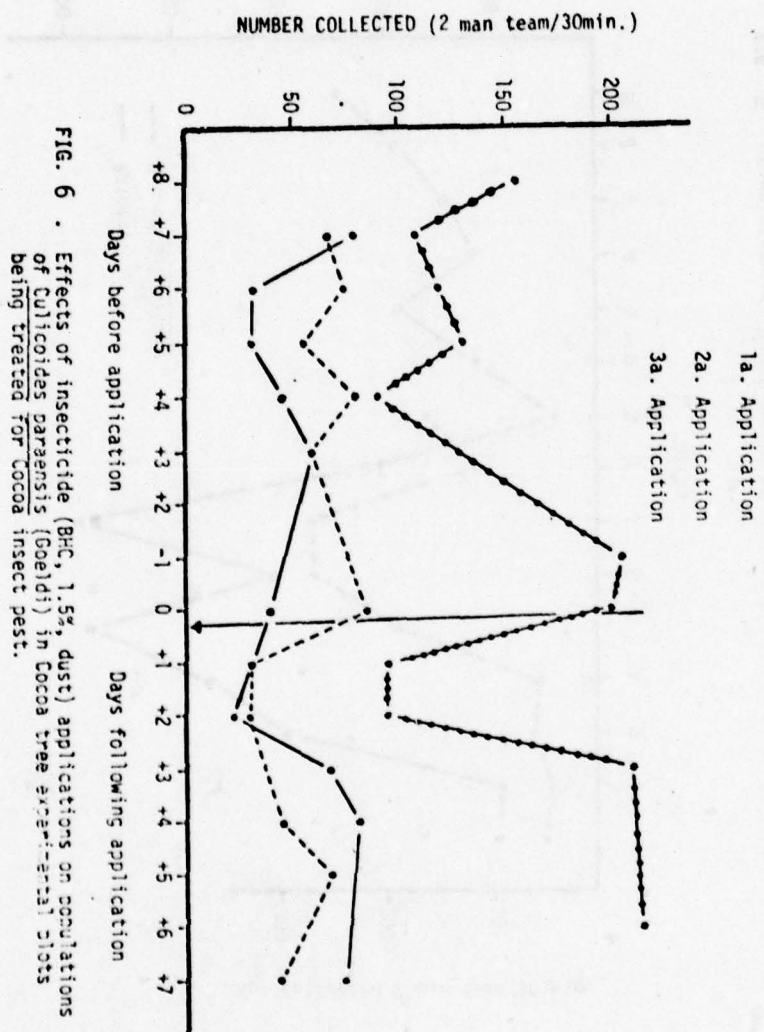


FIG. 6 . Effects of insecticide (BHC, 1.5%, dust) applications on populations of *Culex tarsalis* (Goeldi) in Cocoa tree experimental plots being treated for Cocoa insect pest.

apparent from the available information that decaying banana stalks are the preferred breeding habitat when compared to banana stump habitat. Unfortunately, no behavioral data for C. paraensis is available to explain the distinction of breeding site selection. However, the most important fundamental fact is that C. paraensis readily breeds in the decaying banana tree substrate.

Decaying cacao hulls were also demonstrated to be a preferred breeding site for several species of midges and C. paraensis. When the cacao pod is processed manually, the pod is divided into two parts to facilitate the removal of the cacao seeds. Thus the divided hulls form artificial containers for breeding sites. However, it has been observed that some organic decomposition must occur before breeding in the highly organic substrate is possible.

It has been a cultural practice for many Brazilians in the Amazon region to plant banana trees near their residences. Within the urban environment it is quite common to observe numerous small groups of banana trees associated with the residences. Banana trees are maintained near the residence for various reasons. Normally, banana trees when planted in small groups near the houses provide a source of fruit which supplements the family diet; however, it has also been observed in numerous cases that banana trees are maintained to provide shade or to prevent soil erosion.

When a banana tree yields fruit, it is common practice to harvest the banana cluster by cutting the entire trunk on which the fruit was borne, since this portion of the tree will not bear fruit again. Normally, the tree trunk is cut into several smaller sections (stalks) and left at the base of the remaining tree. It is important to note that sectioning the banana tree trunk increases the available breeding material, since egg oviposition and larval development occurs primarily on the exposed ends of the stalks and the decaying material within. Banana stalks form a decaying substrate which is utilized as an organic fertilizer. The length of time required for decomposition of the banana substrate is variable depending on environmental conditions; however, the substrate will normally serve as a breeding site for midges for 2 to 4 months.

The second preferred breeding habitat associated with cultural practices in the Amazon basin is that of cacao production. Normally, cacao plantations are not closely associated with larger urban areas; however, large plantations of cacao have been observed to be closely associated with smaller agriculturally oriented urban areas. Cacao is normally harvested once a year during the months of January-March. Normally, after the cacao pods are removed from the tree they are taken to a central area, usually near the owners residence, and processed for the seed from which cocoa is derived. This process is performed by manual labor which requires the cacao

pods to be opened and the seeds extracted. The problem that is then derived from the processing is proper disposal of large volumes of cacao hulls which have no commercial value. Normally, the cacao hulls are piled in large mounds and left to decompose naturally. As the cacao substrate is decomposing it provides an excellent breeding habitat for C. paraensis and other midge species. Depending on the quantity of material present, the hulls may produce thousands of man-biting midges.

Since available data indicate that the breeding sites for C. paraensis are closely associated with cultural practices of man, then the problem arises, what are the best methods for reducing or controlling the midge population. Since decomposing banana vegetative matter has been found to be a preferred midge breeding site, it appears that urban populations of C. paraensis could be significantly reduced if the decomposing banana material could be removed from the residential areas. This could be accomplished by simple burial of the banana breeding materials within the area where banana trees are grown or by an organized municipal program in which the material is disposed of in a sanitary landfill. Either method would isolate the material from serving as a breeding site. However, cultural modification within the urban environment cannot be accomplished unless a majority of the residences participate in the control program. It is conceivable that by simple cultural methods that the urban population of C. paraensis could be reduced to a population level below that required for transmission of ORO virus.

Cultural control efforts in areas where there exist large cacao plantations share similar problems to those noted in the urban areas, namely the removal or destruction of the midge breeding sites. Studies conducted in a cacao plantation environment indicate that the decomposing cacao pods is the only substrate producing large numbers of C. paraensis. Therefore, if the cacao pods were disposed in a proper manner this breeding habitat could be significantly reduced. Unfortunately, it is common practice to leave piles of this material near the residence, thereby creating a favorable association of human blood meal and midge breeding sites.

In the event that cultural modification programs are ineffective, then temporary control programs with the use of insecticide would have to be initiated. Diurnal activity patterns for C. paraensis described previously indicate that the biting midge population is most active during the latter evening hours (16:00-18:00 hrs). Therefore, it is only reasonable to conclude that a cost-effective pesticide control program would take advantage of this information and concentrate control efforts during this period.

In summary, it would appear that basic modifications in the

cultural practices associated with the proper management of potential breeding materials for *C. paraensis* could significantly reduce the potential for occurrence of ORO epidemics in the Amazon Basin.

2. Man as the principal vertebrate host

OBJECTIVE: The objective of this section is to summarize available information regarding human infection with Oropouche (ORO) virus. Special areas of consideration will be the clinical syndrome and viremia produced following infection. The underlying question is: Can man serve as the principal vertebrate amplifying host in the epidemic cycle of ORO virus?

BACKGROUND: The previous sections have clearly established that Culicoides paraensis is in all probability the primary epidemic vector of ORO virus to man. The question remains though, where the infected vector acquires its infection. The sections on the basic biology of C. paraensis have demonstrated that this species is intimately linked to man through its utilization of breeding sites created by man, and in all probability by using man as its prime source of blood meals. The behavioral patterns described for this species indicate that it utilizes a diurnal activity period and that it is not adverse to entering houses in search of its hosts. Certainly the vector is adequately exposed to man.

The previous section which dealt with ORO virus transmission studies gives some idea as to the threshold of viremia needed to infect feeding C. paraensis. While a definite critical threshold was not established, many C. paraensis were infected when fed on a viremic hamster which titered $10^{5.0}$ SMLD₅₀/0.02 ml, and most of those infected went on to transmit ORO virus by bite to susceptible hamsters. Consequently, one might assume that a viremia titer of this magnitude would be sufficient to serve to infect feeding vectors.

DESCRIPTION: Information presented in this section has been taken from the published references cited. No new studies were conducted.

PROGRESS: Clinical disease in man due to infection with ORO virus was described in detailed by Pinheiro et al. (1976). They reported on an epidemic which occurred in the village of Mojui dos Campos, Pará, Brazil during February, 1975. Clinical symptoms of 68 patients naturally infected with ORO virus during this outbreak are summarized in Table 7. Most frequently reported clinical manifestations were fever, headache, chills and myalgia. Illness was reported to last from 2 to 7 days, and several patients became severely ill, occasionally to the point of prostration.

Titration of ORO virus isolated from naturally infected viremic patients have been reported on occasion. Pinheiro et al. (1962)

COMMENTS: When reviewing population activity of C. paraensis in the domestic environs, it is apparent that the behavioral biting patterns were markedly different between sites, and that monitored physical parameters were also variable between environs. With the available data it is difficult to correlate biting activity with the physical parameters, since several factors affecting biting activity were not evaluated (ex. physiological status of the biting midge, influence of light intensity and duration). However, some beneficial information can be derived from the data.

It is apparent from the large number of Culicoides collected in the domestic environs, that this species would be a good suspect for disease transmission. It is strongly endophilic and readily feeds on man. The data shows that biting activity in the house and shaded collecting sites remained fairly high throughout the day. This behavioral activity is important since it is also human and animal behavior to seek protected areas from the sun and higher temperatures during midday. In addition, this activity pattern suggests that the physical parameters and humidity may be involved in modifying the temporal activity pattern of C. paraensis.

A third parameter, which was not monitored but which is believed to have a major effect on midge population activity, is light intensity. It was noted that the early morning peak in biting activity began with the rapid change in light intensity; however, during this transition phase the physical parameters of temperature and humidity were stable.

The higher number of midges collected in areas showing lower temperatures and higher humidities during midday tends to suggest that the Culicoides are leaving those areas of higher temperatures and concentrating in more protected areas. However, when environmental conditions become more favorable late in the afternoon, between 16:00-18:00 hours, the midge activity markedly increases and disperses.

Table 5 . Observations on habitat preference of *Culicoides paraensis* evaluated by emergence traps. *Culicoides* species were collected by means of emergent traps, Belém, Pará, Brazil, 1977-1978.

Month	Habitat Type					
	Decaying Banana Stalks		Decaying Cacao Hulls		Decaying Banana Stumps	
	No. of T-Days	No. of C.p./ T-Day	No. of T-Days	No. of C.p./ T-Day	No. of T-Days	No. of C.p./ T-Day
June 77	-	-	54	2.18	782	.01
July 77	-	-	62	0.87	850	.01
August 77	-	-	78	0.29	290	.01
September 77	89	16.03	84	7.38	300	.10
October 77	110	8.44	90	7.75	280	.06
November 77	114	11.66	87	3.82	280	.04
December 77	96	4.47	54	6.96	280	.08
January 78	168	27.75	96	7.80	310	.44
February 78	186	2.28	102	19.40	280	.00
March 78	168	6.66	91	17.80	350	.17
April 78	112	3.12	56	11.00	310	.00
May 78	128	2.28	62	1.60	210	.07
June 78	112	.05	66	1.20	320	.02
July 78	124	.83	62	.05	280	.02

Table 5 . Observations of habitat preference of *Culicoides paraensis* evaluated by emergence trap. *Culicoides* species were collected by means of emergent traps, Belém, Pará, Brazil, 1977-1978. - Cont.

Month	Habitat Type			
	Decaying leaf-litter		Marsh land Area	
	No. of T-Days	No. of <i>C. p.</i> /T-Day	No. of T-Days	No. of <i>C. p.</i> /T-Day
June 77	1222	0	-	-
July 77	1316	0	-	-
August 77	1363	0	-	-
September 77	600	0	216	0
October 77	620	0	240	0
November 77	620	0	126	0
December 77	620	0	204	0
January 78	480	0	168	0
February 78	700	0	138	0
March 78	-	-	234	0

/zcm.

Table 6. Quantitative composition of the *Culicoides* fauna* represented by *Culicoides paraensis* determined in two principal breeding habits. *Culicoides* species were collected by means of emergent traps, Belém, Pará, Brazil, 1977-1978.

	Decaying Banana Stalks		Decaying Cocoa Hulls		Habitat Type	
	No. of <i>Culicoides</i> species collected		No. of <i>Culicoides</i> species collected		No. of <i>Culicoides</i> species collected	
	No. C. P.	% C. P.	No. C. P.	% C. P.	No. C. P.	% C. P.
June 77	-	-	-	-	118	2.0
July 77	-	-	7156	-	54	1.0
August 77	-	-	5158	-	23	1.0
September 77	1473	97.0	3449	97.0	620	8.0
October 77	933	99.0	8541	99.0	698	7.0
November 77	1788	99.0	9351	99.0	332	3.0
December 77	474	70.0	11079	70.0	376	9.0
January 78	7121	65.0	4027	65.0	746	6.5
February 78	880	48.2	11524	48.2	1977	10.9
March 78	2410	46.5	18103	46.5	1625	9.7
April 78	1479	23.6	16674	23.6	618	12.4
May 78	829	35.2	4967	35.2	99	4.5
June 78	266	2.2	2213	2.2	79	2.1
July 78	1606	12.8	3838	12.8	3	< .1
			2567			

* Species of *Culicoides* and *Forcipomyia* spp collected

<i>C. paraensis</i>	<i>C. insinuatus</i>	<i>C. hylas</i>
<i>C. debilipalpis</i>	<i>C. tetrathynia</i>	<i>Forcipomyia</i> spp.
<i>C. fusipalpis</i>	<i>C. foxi</i>	

/zcm.

presented titers of 15 patients bled during illness and showed a maximum titer of $10^{4.8}$ SMLD₅₀/0.02 ml. Five (33.3%) of the fifteen sera tested, had titers equal to or greater than $10^{4.0}$ SMLD₅₀/0.02 ml. These results are reproduced in Table 8. In the Mojui dos Campos study, viremia was titrated in 6 patients, and results of comparative titration methods were also reported. In this study, 5 (83%) of the 6 sera tested titrated equal to or greater than $10^{4.0}$ SMLD₅₀/0.02 ml, and the highest titer recorded was $10^{5.2}$ SMLD₅₀/0.02 ml. These results are reproduced in Table 9. A seventh patient, not included in Table 9, was reported with a maximum titer of $10^{6.0}$ SMLD₅₀/0.02 ml. A patient in whom viremia was followed daily was viremic during the first 4 days of illness, but negative on the 5th day.

COMMENT: Results summarized here clearly indicate that man, when infected with ORO virus, produces a viremia of sufficient titer to infect at least a portion of the feeding *C. paraensis*. If $10^{5.0}$ SMLD₅₀/0.02 ml is used as a critical threshold to infect feeding midges, then 2 (9%) of the 22 reported cases could serve as amplifying hosts. If $10^{4.0}$ SMLD₅₀/0.02 ml is considered the critical threshold, then 11 (50%) of the 22 patients tested could contribute. With a viremic period which spans 4 days, and apparently sufficient viremia titers to infect feeding vectors produced during at least part of the that time, it seems probable that man is the principal vertebrate amplifying host in the epidemic cycle of ORO virus. The fact that man may become quite ill with ORO virus infection, to the point of remaining at home in bed, does not lessen the impact of his potential contribution, since it has already shown that *C. paraensis* will quite readily enter houses in search of human blood meals.

LITERATURE CITED

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2. Pinheiro, F. P., Travassos da Rosa, A. P. A., Travassos da Rosa, J. F. and Bensabath, G.: An outbreak of Oropouche virus disease in the vicinity of Santarém, Pará, Brazil. Tropenmed. Parasit. 27: 213-223, 1976.

TABLE 7. Clinical symptoms of 68 patients infected with Oropouche virus during an outbreak in Mojui dos Campos, Pará, Brazil, 1975*.

Signs or Symptoms	%
Fever	97
Headache	88
Chills	85
Myalgia	82
Arthralgia	67
Photophobia	66
Dizziness	63
Nausea	26
Conjunctival congestion	22
Vomiting	17
Diarrhea	13
Coryza	3
Cough	3

* From Pinheiro, et al., 1976.

TABLE 8. Summary of Oropouche (ORO) viremia titers among 15 patients from whom the virus was isolated during an epidemic in Belém, Pará, Brazil; April and May, 1961*.

Specimen number	Titer of ORO virus in sera
H 29604	4.8 log ₁₀ SMLD ₅₀ /0.02 ml
H 29086	4.7
H 29716	4.5
H 29762	4.0; 3.0
H 30125	4.0
H 29327	3.9
H 29020	3.8
H 29516	3.5
H 29717	3.4
H 29872	3.4
H 30079	3.2
H 29329	2.9
H 29761	2.4
H 29559	1.8
H 29718	0.8

* From Pinheiro et al., 1962.

TABLE 9. Summary of Oropouche viremia titers among 6 patients infected during an epidemic in Mojui dos Campos, Pará, Brazil in 1975. Also shown is a comparison of the sensitivity of different methods for titrating Oropouche virus*.

Specimen Numbers	Vero cells			Baby mice ic LD ₅₀ /0.02 ml
	PFU/0.1 ml	TCD ₅₀ /0.01 ml		
		Tubes	Microplates	
H 275932	4.9 **	5.5	-	4.3
H 273999	4.5	4.5	-	4.0
H 274000	-	4.7	-	3.5
H 274053	-	3.5	3.5	4.0
H 274055	5.7	6.0	5.0	5.2
H 274082	4.6	-	4.5	4.8

* From Pinheiro et al., 1976.

** Log₁₀

3. The Tomé Açú epidemic

OBJECTIVE: The objective of this section is to conduct concurrent epidemiological and entomological surveys during an Oropouche (ORO) virus epidemic in Tomé Açú and contiguous rural settlements. Additional objectives of the survey were to:

- a. determine the prevalence of ORO virus in the inhabitants of Tomé Açú and surrounding rural areas.
- b. define the geographical distribution of clinical cases of ORO virus.
- c. quantify and identify the hematophagous insects biting man in a peridomestic environment.
- d. identify the epidemic vector of ORO virus.

BACKGROUND: During the second quarter of 1978, reports of clinical cases of Yellow Fever (YF) virus from Tomé Açú area were referred to the Institute Evandro Chagas virology section. In August, members of the virology section and USAMRU traveled to the areas where YF virus was reported. During a general epidemiological survey of the inhabitants in the area, the Tomé Açú hospital was visited to determine if febrile patients were present. An adolescent female patient had been admitted on the day of the visit with clinical signs of fever and general discomfort. Blood was drawn for virus isolation attempt. The virus isolated was identified as ORO virus. Thus, it became apparent that two epidemics of arboviral agents were occurring concurrently, both YF and ORO. Subsequently, field investigations for ORO virus activity in the Tomé Açú area were initiated by a joint effort of Institute Evandro Chagas and USAMRU.

DESCRIPTION: Tomé Açú is a small village located due south of Belem in the state of Pará. The area of Tomé Açú is divided into various jurisdictional areas, in which the village of Quatro Bocas lies. Early clinical cases of ORO virus were reported primarily from Quatro Bocas. From early case reports it was felt that the disease focus was located in the vicinity of Quatro Bocas. This village serves as a commercial center for the large agriculture area surrounding Quatro Bocas.

Epidemiological efforts were concentrated within the village and nearby agriculture farms; however, reports of persons with fever in nearby settlements were also investigated when feasible. A

house-to-house survey of Quatro Bocas to identify those persons exhibiting clinical signs of ORO virus infection was conducted by a 2 man epidemiological survey team. Case histories were recorded and blood samples were drawn for virus isolation attempts. Blood samples were stored in liquid nitrogen and transferred to the Institute Evandro Chagas for virus isolation attempts. White mice and hamsters were utilized to isolate ORO virus from febrile cases.

Entomological surveys for hematophagous insects were conducted primarily in two areas of the village and two peripheral areas. Capture sites are shown in Figure 7. Sites were chosen based on epidemiological evidence of ORO virus activity in those areas. Systematic man-biting captures for insects were performed by two man teams making collections near the houses. Night time man-biting collections and CDC light traps baited with carbon dioxide were used. Captured hematophagous insects were gathered periodically and transferred to the field laboratory where they were separated into general taxonomic groups and subdivided into blood fed and non-engorged. Only non-engorged insects were tested for virus isolation. Field material was conserved in liquid nitrogen and returned to the main laboratory for virus isolations attempts.

In Belém, collected insects were identified and pooled for virus isolation attempts. Initial pool size for Culicoides paraensis was approximately 100 individuals. When it later became apparent that several thousand Culicoides would need to be tested, the pool size was raised to 200.

Culicoides were assayed for virus in Vero cells grown in tubes. Pools of Culicoides were triturated with tissue grinders in 1.0 ml of 25% Bovine plasma albumin in phosphate buffered saline with antibodies. Triturated pools were centrifuged at low speeds for 15 minutes, then 0.1 ml of supernatant fluids inoculated into duplicate drained tubes of Vero cells. Tubes were then incubated for 1 hr at 37°C, rinsed, and 1.0 ml of fresh maintenance media added. Tubes were observed daily for evidence of viral cytopathic effect (CPE) for 15 days post inoculation. Oropouche virus normally causes CPE on initial passage in Vero cells between days 10 and 14.

PROGRESS: Table 10 presents a summary of the age, sex, occupation and area of residence for confirmed cases of ORO virus in the area of Tomé Açú.

During the initial ORO virus survey in the Tomé Açú area, 20 strains of ORO virus were isolated from human blood samples. Twelve of the isolates were from persons residing within the village of Quatro Bocas, with one case occurring on cacao plantations bordering the village. One case was identified in Tomé Açú, which is located approximately 13 kilometers west of Quatro

Bocas. Two cases were reported from Arraia, a small village located to the north of Quatro Bocas. Four additional cases were recorded from field technicians investigating the epidemic in Quatro Bocas and one case recorded from a female visiting the area during the epidemic.

Entomological survey results show that 15 species of insects were collected by man-biting captures in the village of Quatro Bocas (Table 11). Capture data shows that the dominate diurnal peridomiciliary species was C. paraensis, with Culex fatigans and Culex coronator being the dominate nocturnal species. Numbers of hematophagous insects collected during the survey were fairly variable when comparing spatial distribution of insects within the village and surrounding agriculture areas. The greatest difference noted in spatial distribution of insects was for C. paraensis, the suspect vector of ORO virus. The largest number of C. paraensis recorded was associated with those areas involved in cacao production, with the fewest numbers being collected from bordering areas.

Figure 8 shows the diurnal biting pattern for C. paraensis near a house located on a cacao plantation. This area was found to have a large population of biting midges. This biting activity was found to be a trimodal pattern with the highest activity occurring between 16:00 and 18:00 hours.

Preliminary results are available for virus isolation attempts from insects collected from Quatro Bocas. To date a total of 155 pools representing 24,465 individuals of C. paraensis have been tested for virus in cell culture. Of these, 4 pools have produced CPE characteristic of ORO virus. All 4 have been successfully passed in cell culture, all are neutralized by antibody specific to ORO virus. All 4 pools were collected from the same locality, a Japanese cacao plantation which had several persons ill with ORO virus infection.

Figure 9 graphically depicts the location of residencies for 12 persons from whom ORO virus was isolated. In addition, the collection site of the 4 pools of C. paraensis from which ORO virus was isolated is also presented. Clearly ORO virus activity was dispersed throughout the village of Quatro Bocas, and not localized in any particular area.

COMMENTS: Epidemiological findings indicate that an epidemic of ORO virus was being experienced by the inhabitants of the Tomé Açu area, with an apparent focus of disease activity occurring in Quatro Bocas and adjoining agricultural farms. However, data is not available to determine the initial onset of the disease, since it is a common practice in small communities of the Amazon Basin for ill persons to remain in the home and not seek medical attention.

It was apparent from the preliminary epidemiological findings that ORO virus was more prevalent among the inhabitants than was indicated by the number of confirmed cases. When the house-to-house survey was being conducted, numerous households indicated that febrile symptoms similar to ORO virus disease had been experienced by family members; however, the etiological disease was unknown. It would be reasonable to assume that a proportion of these febrile reports could be attributed to ORO virus. Due to the general dispersion of ORO virus isolates within the village, it was not felt that the disease prevalence could be associated with a particular residential area (Figure 9). This was expected since the village area is small and the inhabitants are quite transient.

From the available data derived from the case histories of confirmed ORO virus isolates, it was found that 85% (17/20) of the cases were males with an age range of 7 to 58. Five of the male cases were persons associated with agriculture related work, and since cacao is one of the main agriculture crops, it can be assumed that these individuals were working in areas of high populations of Culicoides. Only 15% (3/20) of the cases were female, thus the data tends to suggest that activities or occupations are important factors in determining the risk of exposure to ORO virus infection.

It should be noted that the residential location was an important factor when considering the spatial distribution of hematophagous insects (Table 11). An approximately two fold variation was noted for Culex fatigans and Culex coronator in two areas of the village. However, the most marked differences was noted for C. paraensis.

Since the epidemiological and laboratory transmission data accumulated to date indicates that Culicoides paraensis is the most probable insect vector, it is important to note that this species was the dominate species collected in all capture areas. However, the number of C. paraensis was found to be considerably higher in the agriculture areas producing cacao than in the village. These findings were not unexpected, since prior studies at a cacao research station in Belém demonstrated that rotting cacao husk provides a natural breeding substrate for several species of midges including that of C. paraensis. Samples of the cacao husks collected at the cacao plantations yielded several individuals of C. paraensis. Since Quatro Bocas is primarily an agricultural center, several large cacao plantations border the small village; therefore, it is proposed that main population of C. paraensis observed within the village can be attributed to the dispersion of this biting midge from nearby cacao plantations. The other recognized breeding site for C. paraensis, decomposing banana tree substrate, does not appear to be important in Quatro Bocas since few banana trees were observed within the village.



Areas where man biting insects were collected.

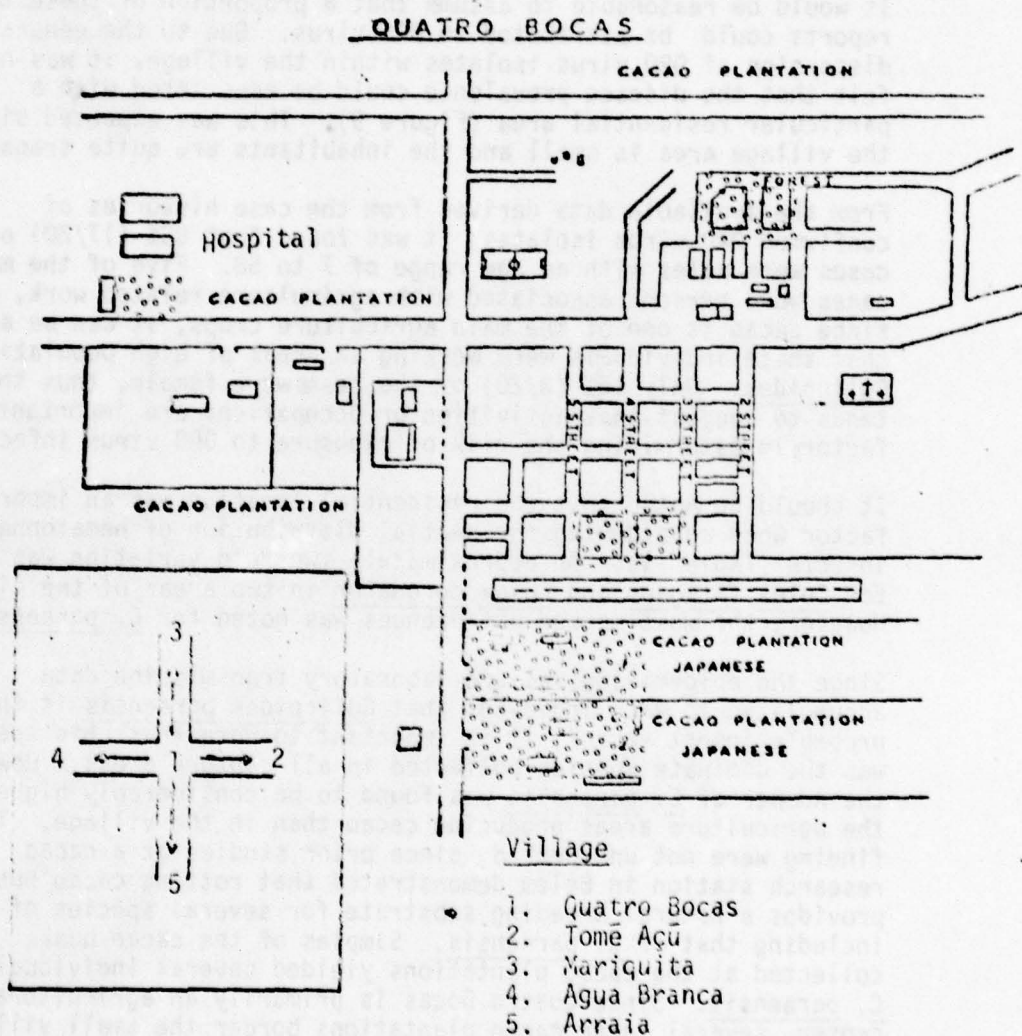


Fig. 7. Spatial distribution of capture sites in Quatro Bocas, Pará, Brazil, 1978.

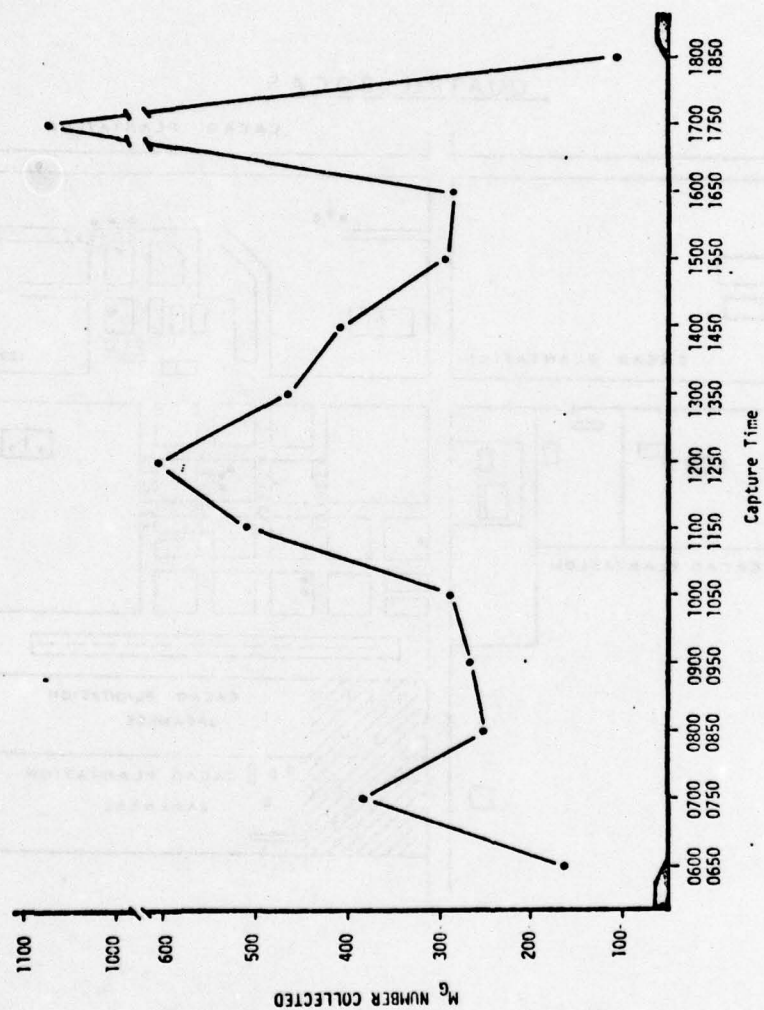


FIG. 8. Peridomestic diurnal activity pattern for *Culicoides paraensis* (Goeldi) in Quatro Bocas, Pará, Brazil, 1978



Area in which four strains of Oropouche virus was isolated from pools of C. paraensis.

- Spatial distribution of Oropouche virus isolates from man.

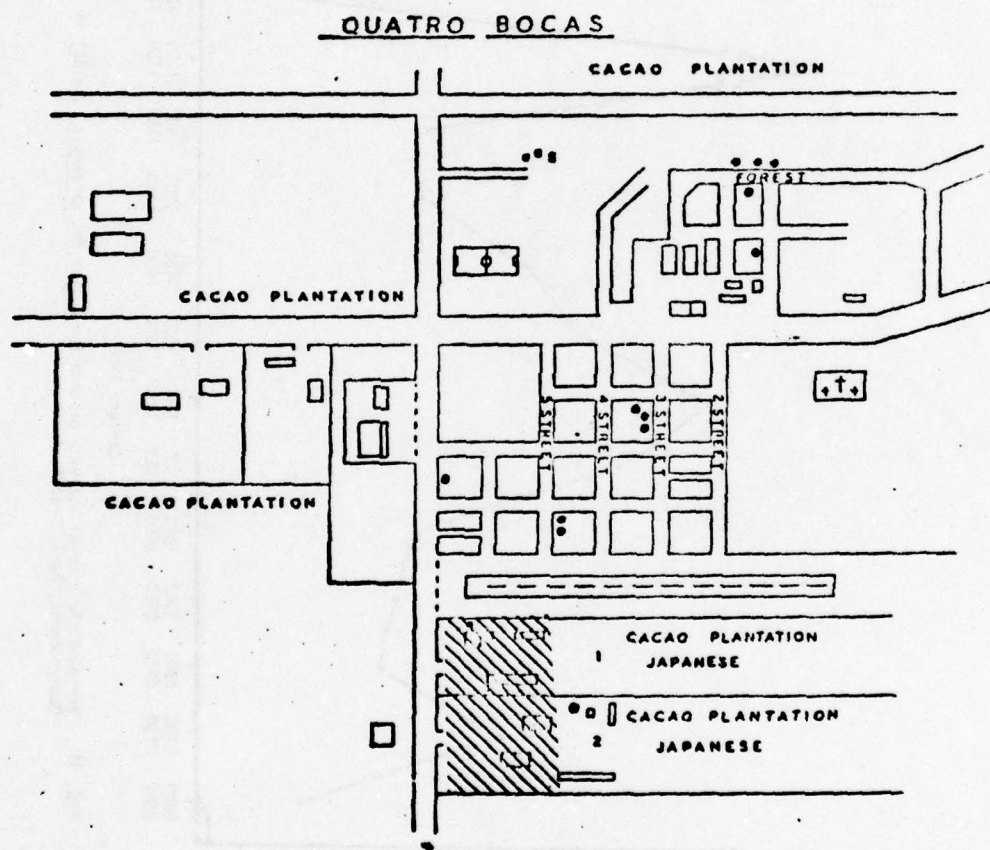


Fig. 9. Spatial distribution of Oropouche virus isolates from man and C. paraensis in Quatro Boca, Pará, Brazil, 1978.

TABLE 10. Age, sex and occupation data for Oropouche cases determined by virus isolation.

City of residence	Sex		Age	Occupation
	M	F		
Quatro Bocas	X		58	Agriculture laborer
Quatro Bocas	X		19	Unknown
Quatro Bocas	X		6	Unknown
Quatro Bocas	X		10	Student
Quatro Bocas	X		14	Unknown
Quatro Bocas	X		7	Student
Quatro Bocas	X		43	Agriculture laborer
Quatro Bocas	X		59	House construction worker
Quatro Bocas	X		9	Student
Quatro Bocas	X		20	City water works
Arraia	X		18	Agriculture laborer
Arraia	X		15	Agriculture laborer
Tomé Açú	X		28	Agriculture engineer, cacao
Belém*	X		27	Field technician*
Belém*	X		16	Field collector*
Belém*	X		27	Laboratory technician*
Belém*	X		15	Field collector*
Quatro Bocas		X	22	Store clerk
Quatro Bocas		X	23	Store clerk
Belém		X	27	Visitor

* Members of field teams investigating the epidemic

Table 11. List of hematophagous insects collected in an initial survey of Quatro Bocas during an Oropouche virus epidemic, Pará, Brazil, 1978

Location and species collected	Diurnal man-biting captures* (peridomiliary)	Nocturnal captures		Total
		Man-biting	CDC**	
Forest area				
<u>Culicoides paraensis</u>	171	0	0	171
<u>Aedes scapularis</u>	1	0	0	1
<u>Culex fatigans</u>	14	67	7	88
<u>Culex coronator</u>	18	15	16	49
<u>Culex declarator</u>	9	0	17	26
<u>Culex corniger</u>	10	0	2	12
<u>Limatus durhamii</u>	2	0	0	2
<u>Psorophora cingulata</u>	0	2	0	2
<u>Trichoprosopon digitatum</u>	1	0	0	1
<u>Wyeomyia spp</u>	4	0	0	4
Village Street # 5				
<u>Culicoides paraensis</u>	652	0	0	652
<u>Aedes scapularis</u>	0	1	1	2
<u>Anopheles (Nys) nuneztovari</u>	0			
<u>Culex fatigans</u>	22	111	39	172
<u>Culex coronator</u>	42	4	29	75
<u>Culex Sp # 21</u>	0	0	1	1
<u>Culex declarator</u>	0	4	0	4
<u>Culex (Mel.) sp</u>	1	0	0	1
<u>Culex corniger</u>	5	0	5	10
<u>Culex spp</u>	0	3	4	7
<u>Psorophora ferox</u>	2	0	0	2
<u>Psorophora cingulata</u>	0	2	5	7
Japanese Cacao Plantation				
<u>Culicoides paraensis</u>	5056	NT***	NT	5056
Hospital				
<u>Culicoides paraensis</u>	1001	NT	NT	1001

* Captures times were 0700 to 1850 hrs.

** Light traps were operated from 1900 to 0700 hrs.

*** Nocturnal captures were not preformed.

B. Studies on the Endemic Cycle

1. Curuã-Una study

a. Vectors present

OBJECTIVE: The objectives of this study were to conduct field surveillance of hematophagous insects in forested areas in an effort to incriminate natural vector(s) of Oropouche virus, and when possible, to determine seasonal and ecological associations of potential insect vectors.

BACKGROUND: In May 1977 an entomological and ecological field surveillance program was initiated in Curuã-Una to investigate Oropouche (ORO) virus activity in a sylvatic environment. Curuã-Una is located approximately 44 km south and 40 km west of Santarém, Pará, Brazil. This area was selected for study because in 1975 an epidemic of ORO virus was investigated in a small village, Mojui dos Campos, which lies near the Curuã-Una forest. It was felt that perhaps ORO virus was endemic in this forested area, and that ORO virus may have been introduced into Mojui dos Campos from it.

Available epidemiological and entomological information tends to support the hypothesis that the disease is maintained in a sylvatic vertebrate-insect cycle of transmission. This theory is supported by serological evidence of ORO virus activity among forest inhabiting birds and mammals.

DESCRIPTION: The field surveillance program was designed to monitor hematophagous insects for virus activity and to obtain basic entomological information concerning insect ecology. Entomological information was gathered by multiple collecting techniques (light traps, Shannon trap, and man biting collections). A routine ground and canopy collecting program was established at three separate field collecting stations (Figure 10). The systematic collecting program was designed to study both the diurnally and nocturnally feeding insect activity. All medically important insects collected are in the process of being classified and assayed for virus.

A sentinel hamster animal program was also conducted for a short period of time during the surveillance. Sentinel animals were marked by digital clipping and exposed at two forest levels, ground and canopy. Hamsters exposed during the diurnal period (06:00-18:00 hrs.) were replaced by a second group of hamsters which were exposed during the nocturnal period (18:00-06:00 hrs.). After being exposed for 2 weeks in the sylvatic environment, they were maintained in the laboratory for two additional weeks and observed

for overt clinical signs of virus activity. Subsequently, they were sacrificed for serological testing for virus antibodies.

The ecological descriptions for the study area are included in a subsequent section of this report.

PROGRESS: The sylvatic surveillance program was concluded after one season of observations. However, material from this investigation is still being identified and processed for virus isolation attempts. Therefore, only general results will be presented here.

Table 12 presents a preliminary list of hematophagous insects collected by various collecting methods at the three Curuã-Una forested collecting stations. To date, 43 species of Culicidae have been identified and 7 species of biting midges (Ceratopogonidae). The largest number of hematophagous insects collected belong to the Culicinae and Phlebotomus taxonomical groups.

Table 13 summarizes the results of the sentinel hamster program utilized to survey sylvatic virus activity. Seventy nine (79) hamsters were exposed for 6 days each. Only one isolation of virus was obtained, Kwatta virus, and although all sera were tested against a block of 19 antigens, no sero-conversions were noted.

Tables 14, 15 and 16 present the monthly geometric mean of the numbers of taxonomical groups (Culicidae, Psychodidae, Ceratopogonidae) of insects by two collecting methods (man biting, shannon trap). Mosquitoes (Culicidae) and sandflies (Psychodidae) were the dominate groups collected.

COMMENTS: As is shown in the species list, the sylvatic environs of Curuã-Una produce a significant number of hematophagous insect species, and thus, demonstrate a wide potential disease vector spectrum. It would therefore seem reasonable to assume that if there exists a sylvatic vector-vertebrate cycle of ORO virus within the study environment, then the basic entomological information obtained during this study would be useful when a vector species is eventually identified. However, to date none of the sylvatic species have been shown to be involved in ORO virus transmission.

The sentinel hamster program resulted in the isolation of Kwatta virus from a hamster exposed at night at ground level. Kwatta virus was first isolated in Surinam from a Culex mosquito pool. Little is known regarding the natural history of the virus. The isolation of the virus here from the sentinel hamster tends to support a theory of vector-host transmission.

Collection data in Tables 14, 15 and 16 demonstrate that the

mosquito fauna represents the largest number of sylvatic hematophagous insects in these areas. Within this group, the number of Culicinae collected were superior to those of the Anophelinae fauna. This was representative of the environment. Ecologically, in areas 1 and 2, the primary breeding sites for Culicinae species were temporary rain water habitats: rain pools, tree holes, fruit pod containers, etc. Breeding habitats for Anopheles were primarily confined to near the river, which was relatively fast flowing and had few aquatic plants, and consequently rather unfavorable for Anopheles.

The collections of Culicines at area 3 were notably higher than the number recorded for areas 1 and 2. This can be attributed to the large number of Culicinae mosquitoes breeding in the near by river habitat. The river near this site expands into a cove which has numerous aquatic plants covering most of the water surface. This type of habitat is quite favorable for the mosquito species of Mansonia and Coquillettidia, which were the dominate genera collected in this area.

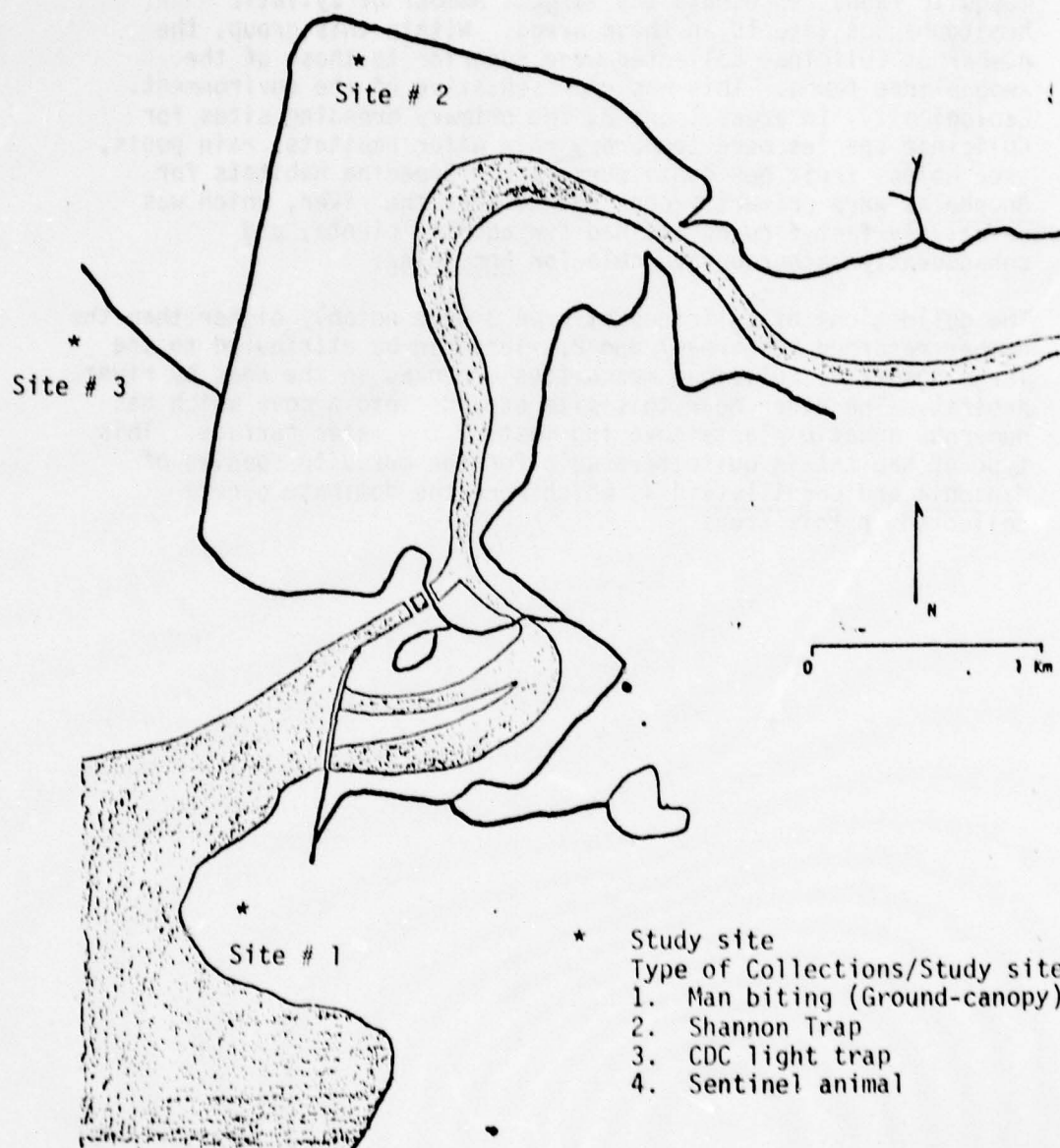


Fig. 10 . Geographical distribution of Entomological study sites in the sylvatic area of Curuá-Una, Pará, Brazil, 1977-1978.

Table 12. Preliminary list of mosquito species collected by multiple collecting methods in forest areas of Curuá-Una, Pará, Brazil, 1977-1978.

<u>Aedeomyia squamipennis</u>	<u>Psorophora (Jan.) albipes</u>
<u>Aedes (Fin.) argyrothorax</u>	<u>Psorophora (Jan.) ferox</u>
<u>Aedes (How.) fluviathorax</u>	<u>Limatus durhamii</u>
<u>Aedes (Fin.) fluviatilis</u>	<u>Limatus flavisetosus</u>
<u>Aedes (Och.) fulvus</u>	<u>Limatus pseudomethysticus</u>
<u>Aedes (Och.) serratus</u>	<u>Sabethes (Sab.) belisarioi</u>
<u>Aedes (How.) septemstriatus</u>	<u>Sabethes (Sab.) cyaneus</u>
<u>Anopheles (Ano.) mattogrossensis</u>	<u>Sabethes (Sab.) shannon</u>
<u>Anopheles (Ano.) mediopunctatus</u>	<u>Sabethes (Sab.) tarsapus</u>
<u>Anopheles (Nys.) albitarsis</u>	<u>Sabethes (Sab.) glaucodaemon</u>
<u>Anopheles (Nys.) nuneztovari</u>	<u>Sabethes (Sab.) chloropterus</u>
<u>Anopheles (Nys.) oswaldoi</u>	<u>Trichoprosopon (Trc.) digitatum</u>
<u>Anopheles (Nys.) triannulatus</u>	<u>Uranotaenia geometrica</u>
<u>Culex sp B 21</u>	<u>Uranotaenia lowii</u>
<u>Culex (Cux.) corniger</u>	<u>Uranotaenia pulcherrimus</u>
<u>Culex (Cux.) coronator</u>	<u>Uranotaenia calosomata</u>
<u>Culex (Cux.) declarator</u>	<u>Wyeomyia (Den.) aporoma</u>
<u>Culex (Mel.) vomerifer</u>	<u>Anopheles (Nys.) sp.</u>
<u>Culex (Mel.) vomerifer complex</u>	<u>Culex spp.</u>
<u>Coquillettidia nigricans</u>	<u>Culex (Carrollia) sp.</u>
<u>Coquillettidia venezuelensis</u>	<u>Culex (Mel.) sp.</u>
<u>Mansonia (Man.) titillans</u>	<u>Haemagogus spp</u>
<u>Mansonia (Man.) pseudotitillans</u>	<u>Psorophora spp.</u>
<u>Mansonia (Man.) amazonensis</u>	<u>Uranotaenia spp.</u>
<u>Orthopodomyia fascipes</u>	<u>Limatus spp.</u>
	<u>Wyeomyia spp.</u>

TABLE 13. Summary of the sentinel hamster surveillance program monitoring virus activity in the Curuā-Una forest study areas, Pará, Brazil, 1978.

Month	Location of sentinel animal - forest level			
	Ground		Canopy	
	Day	Night	Day	Night
November	14*	13**	2	2
December	6	6	2	2
January	8	8	2	2
February	4	4	2	2

* Kwatta virus was isolated from one sentinel hamster.

** Number of sentinel animals monitored for 6 day exposure.

b. Vertebrate host serology

OBJECTIVE: Objectives of the program are to determine which species of mammals and birds may serve as a reservoir to Oropouche (ORO) virus in a sylvatic environment, and to elucidate their role in the disease cycle. Habitat preferences, breeding cycles, and annual population fluctuations of incriminated species will also be documented.

BACKGROUND: Antibody to ORO virus has been found in sera of various mammals and birds. The only recorded isolation from a non-human vertebrate was from a three-toed sloth (*Bradypus variegatus*) collected 150 km southeast of Belém. As part of the general study of the ecology of ORO virus, mammals and birds were collected by trapping, hunting and mist-netting from 15 June 1977 through 10 February 1978 in the Curuá-Una study area.

DESCRIPTION: The Curuá-Una study site is located 44 km south and 40 km west of Santarém, Pará at the Curuá-Una Hydroelectric Plant (latitude 2°50'S, longitude 54°22'W). Mammals and birds were collected in the forest on both sides of the Curuá-Una River which flows from west to east and is approximately 100 m wide. The general topography of the area ranged from flatland to steep slopes. The river below the hydroelectric plant is 47 m above sea level and the hills rise to approximately 100 m. The forest is classified as a tropical semi-evergreen seasonal forest (Beard, 1944). The soil ranges from a reddish-brown sandy clay to clay-sand. The A₁ horizon (humus layer) varies from 1 to 2 cm in thickness and contains many fine hair-like roots. The A₀ horizon (litter layer) is from 4 to 7 cm thick and composed of fallen leaves and twigs. Moss cover on fallen trees and exposed rocks is common. A shrub layer of thin woody plants, 0.5 to 3 m tall is fairly thick, but it is not necessary to cut a trail to walk through the forest. Small trees and palms, to 10 m in height make up an open canopy. Larger trees, from 15 to 20 m tall with slim trunks, are common, and the emergents to 30 m in height are scattered throughout. Small scattered thickets of thin stemmed bamboo are also present. Epiphytes are not common, but small vines and lianas are plentiful. Some of the smaller trees have stilted roots, and buttressing is evident on a few of the emergents.

Small mammals were collected in National live traps (150x150x485mm) and Rinker live traps (80x80x255 mm) which were set at 10 m intervals along established trails in the forest. The traps were

Table 14. Seasonal variation of hematophagous insects collected at a field station (Area 1) within the sylvatic area of Curua-Una. Collections were performed by man-biting and Shannon trap. Curua-Una, Para, Brazil, 1977-1978.

Taxonomic Group	JUL 77	AUG 77	SET 77	OCT 77	NOV 77	DEZ 77	JAN 78	FEB 78	MAR 78	APR 78	MAY 78	JUN 78	JUL 78
	Man biting collections - Captures Time (17:30 - 18:30)												
Culicidae (Anophelinae)	2.0	2.8	2.2	1.7	1.3	1.1	1.0	1.1	1.2	1.8	2.5	1.9	2.5
Culicidae (Culicinae)	10.7	11.7	18.0	19.9	2.3	27.4	29.4	11.6	13.9	10.3	29.2	38.8	36.1
Psychodidae (Phlebotomus)	6.3	5.3	2.8	3.9	1.1	1.8	1.4	1.2	1.6	2.3	2.9	4.3	1.5
Ceratopogonidae (Culicoides)	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.1	1.1	1.0
	Shannon Trap - Capture Time (19:30 - 20:30)												
Culicidae (Anophelinae)	1.5	3.1	1.9	2.4	1.0	2.4	1.2	1.7	1.2	1.6	1.4	2.1	7.3
Culicidae (Culicinae)	9.3	56.1	43.5	29.2	10.0	32.8	19.4	13.3	5.4	10.1	22.7	14.5	47.9
Psychodidae (Phlebotomus)	4.3	5.8	1.1	1.0	2.6	1.0	1.0	1.6	1.3	2.3	2.7	3.1	3.0
Ceratopogonidae (Culicoides)	1.3	1.0	1.0	1.0	1.0	1.0	1.0	1.3	1.0	1.0	1.0	1.5	1.0

Table 15. Seasonal variation of hematophagous insects collected at a field station (Area 2) within the sylvatic area of Curua-Una. Collections were performed by man-biting and Shannon Traps. Curua-Una, Pará, Brazil, 1977 - 1978.

Taxonomic Group	JUL 77	AUG 77	SEP 77	OCT 77	NOV 77	DEC 77	JAN 78	FEB 78	MAR 78	APR 78	MAY 78	JUN 78	JUL 78
	Man biting collections - Captures Time (17:30 - 18:30)												
Culicidae (Anophelinae)	1.2	1.2	1.4	1.1	1.3	1.0	1.4	1.0	1.1	-	1.8	1.0	1.0
Culicidae (Culicinae)	5.3	3.8	4.5	4.3	2.3	1.8	13.5	9.3	6.8	-	10.4	7.4	13.2
Psychodidae (Phlebotomus)	11.9	3.6	1.1	1.1	1.1	1.0	5.1	1.8	13.6	-	4.1	1.6	1.3
Ceratopogonidae (Culicoides)	1.6	1.0	1.0	1.0	1.0	1.0	1.0	1.9	1.4	-	1.3	1.0	1.1
	Shannon Traps - Capture Time (19:30 - 20:30 hrs)												
Culicidae (Anophelinae)	1.2	1.3	1.0	1.4	1.0	1.1	1.7	1.6	1.3	-	1.3	1.2	1.0
Culicidae (Culicinae)	6.7	20.5	58.7	12.1	10.0	9.1	57.2	13.3	8.4	-	8.0	9.4	18.9
Psychodidae (Phlebotomus)	31.3	8.7	1.3	1.1	2.6	1.0	6.6	3.4	4.7	-	14.5	14.5	1.8
Ceratopogonidae (Culicoides)	2.7	1.0	1.0	1.0	1.0	1.0	2.9	1.0	1.9	-	1.6	12.2	1.6

Table 16. Seasonal variation of hematophagous insects collected at a field station (Area 3) within the sylvatic area of Curua-Una. Collections were performed by man-biting and Shannon trap. Curua Una, Pará, Brazil, 1977 - 1978.

Taxonomic Group	JUL 77	AUG 77	SEP 77	OCT 77	NOV 77	DEZ 77	JAN 78	FEB 78	MAR 78	APR 78	MAY 78	JUN 78	JUL 78
Man biting collections - Capture Time (17:30 - 18:30 hrs)													
Culicidae (Anophelinae)	-	-	8.5	6.8	1.8	1.4	1.1	1.0	1.0	-	1.3	1.0	1.0
Culicidae (Culicinae)	-	-	30.5	132.9	29.3	46.1	26.9	30.5	71.2	-	139.6	135.0	79.2
Psychodidae (Phlebotomus)	-	-	1.0	1.0	1.0	1.2	1.0	1.0	1.5	-	1.0	1.1	1.0
Ceratopogonidae (Culicoides)	-	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-	1.0	1.0	1.0
Shannon Trap - Capture Time (19:30 - 20:30 hrs)													
Culicidae (Anophelinae)	-	-	19.9	6.6	4.6	1.7	1.3	1.4	1.0	-	1.2	2.0	4.9
Culicidae (Culicinae)	-	-	91.5	47.5	32.4	34.5	15.8	15.3	15.0	-	99.5	229.0	214.0
Psychodidae (Phlebotomus)	-	-	1.0	1.4	1.1	1.3	1.9	2.0	1.0	-	1.1	1.0	1.0
Ceratopogonidae (Culicoides)	-	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-	1.0	1.0	1.0

left in place until trapping success began to diminish, usually within 2 to 4 weeks. The Rinker traps were also placed in trees to collect arboreal rodents and marsupials.

The traps were placed in the forest described above. Trapping sites 1, 2, 3 and 4 (Figure 11) was in a flat upland forest. Sites 5 and 6 were in an upland forest with gradual slopes. Sites 7 and 8 were in a forest with steep slopes, and sites 9 and 10 were in an upland hillside forest.

The traps were baited with corn and bananas, and checked early in the morning to reduce the number of animals dying. Larger animals, monkeys, sloths, anteaters, etc were hunted. Hunting was conducted both during the night and during the day.

Bats were collected in mist nets, 12 m long and 2.6 m in height, which were usually set across natural flyways through the forest, such as trails and narrow roads (Figure 11). Two mist nets, numbers 17 and 18, were set in a park-like area along the river. The nets were normally tended from dusk until 22:00 hrs. Nets were not opened while the moon was shining brightly.

The mist nets were also set in continuous lines cut through the forest to capture birds (Figure 11). The trail, although kept as narrow as possible, was wide enough to allow passage on both sides of the nets. From 30 to 40 mist nets were opened before dawn and usually tended until 11:00 hrs.

The captured mammals and birds were transferred from the traps and nets to bags and taken to the field laboratory for processing. Each animal was given a collection number at the field laboratory and all specimens taken from it, whole blood, sera, ectoparasites, endoparasites, viscera, etc, were given the same number. After processing in the field lab, all laboratory specimens were preserved and shipped to the base laboratory in Belém. Each animal specimen was preserved either as skin and skull, skull only, or in formalin and shipped to Belém for tentative identification, and later to taxonomists specializing in South American animals to confirm the identification. All information was recorded on field forms which were described in the 1975 Annual Report. A more detailed description of the methods of processing the specimens is in the 1976-1977 Annual Report.

Bird and mammal sera were tested in the Belém laboratory for antibody to viruses by the hemagglutination inhibition (HI) tests. Samples of whole blood and viscera were tested for virus by intracerebral inoculation into suckling mice.

PROGRESS: From 15 June 1977 through 9 February 1978 1,032 mammals representing 62 species, and 777 birds of 26 families were

collected. Three viruses, Urucuri, Flexal and Kwatta, were isolated from these animals. Antibody to ORO, Mayaro and other viruses was found among these sera as well.

Flexal virus was isolated from two species of arboreal rodents. Seventeen percent (2/12) of the Oryzomys bicolor and 25% (2/8) of the O. concolor bloods or viscera tested had Flexal virus. Urucuri virus was isolated from 0.62% (1/162) of the spiny rats, Proechimys longicaudatus (Table 17). Of the 321 Formicariidae tested, Kwatta virus was isolated from one scale-backed antbird, Hylophylax poecilonota (Table 18).

The sera from 824 mammals were tested by HI tests and three sera, two monkey sera and one rodent sera, contained antibody to ORO virus while 11 sera, one marsupial serum, six bat sera and 5 monkey sera, were positive for Mayaro virus (Table 19). Of the 624 bird sera treated with protamine to nullify the influence of heparin and tested by HI tests, 42 sera, three Dendrocolaptidae sera, 35 Formicariidae sera and four Troglodytidae sera, had antibody to ORO virus, while 15 sera, one Dendrocolaptidae serum, 12 Formicariidae sera and two Troglodytidae sera, contained antibodies to Mayaro virus (Table 18).

During the 109 nights of trapping, 348 marsupials and rodents were collected. The spiny rat (Proechimys longicaudatus) was the most commonly trapped mammal (Table 20). The trapping success of the sites north of the river was considerably higher than those south of the river. This may have been due to the northern sites being farther from the hydroelectric plant and the forest less disturbed. Mist nets were tended for 63 nights, and 639 bats were collected (Table 21). Carolla brevicaudata, a fruit eating bat, was the most common bat collected. Forty-three mammals, monkeys, sloths, anteaters, etc, were collected by hunting or by hand.

The bird nets were opened for 59 mornings, and collected 777 specimens. The members of the antbird family, Formicariidae, were the most commonly captured (Table 22). The woodpeckers, Dendrocolaptidae, although half as common as the antbirds, were caught three times more often than any of the remaining families. Bird collecting site B, located north of the river, produced almost twice the amount of birds per morning trapped as either of the two sites south of the river.

COMMENT: Primates, although not common in the Curuā-Una area, had the highest rates of antibody to both ORO virus and Mayaro virus. The two larger species of monkeys, Callicebus torquatus and Alouatta belzebuch, had a higher percent of individuals with antibody than did the marmoset, Callithrix argentata. Very little is known about the longevity of this marmoset in the wild. Although one individual has been reported to have lived for 16

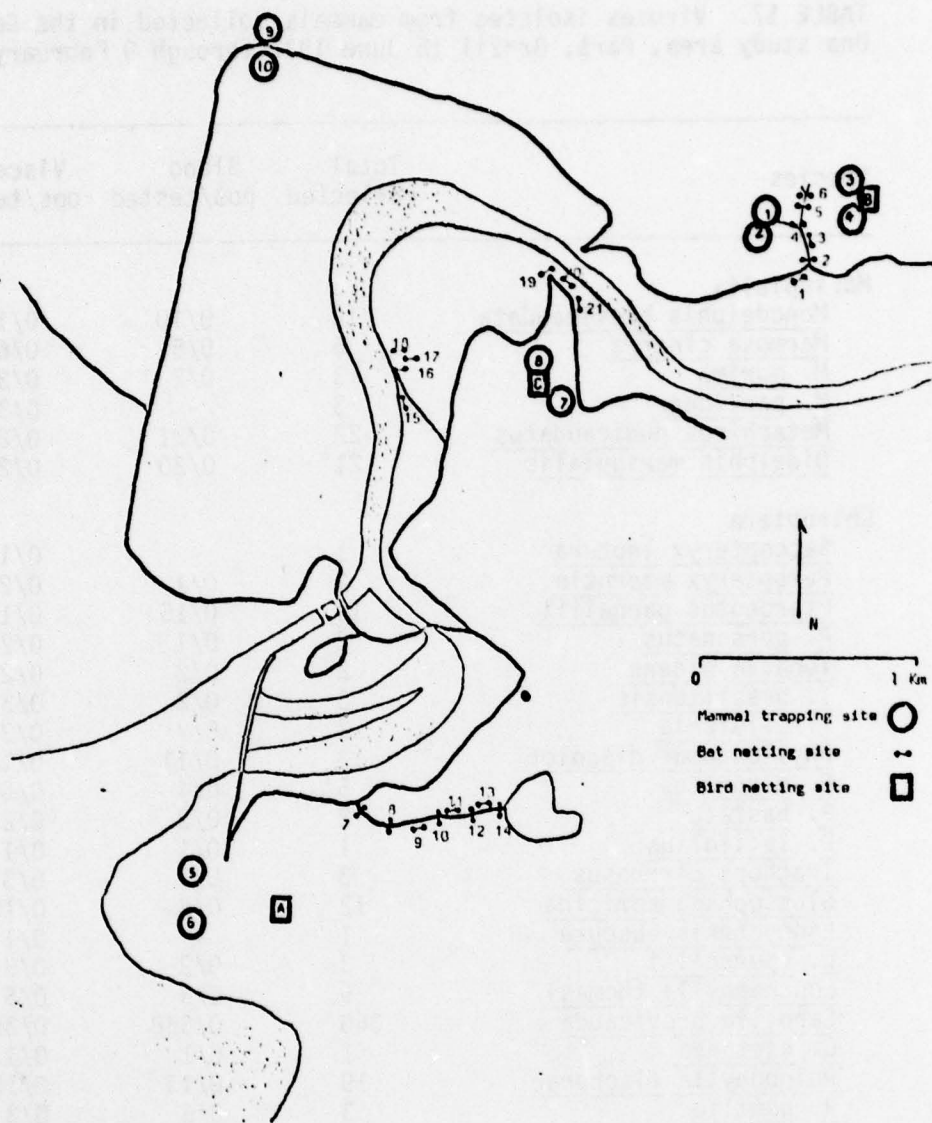


FIG. 11. Mammal and bird collecting sites in the Curuá-Una study area, Pará, Brazil.

TABLE 17. Viruses isolated from mammals collected in the Curuá-Una study area, Pará, Brazil 15 June 1977 through 9 February 1978.

Species	Total collected	Blood pos/tested	Viscera pos/tested
Marsupialia			
<u>Monodelphis brevicaudata</u>	10	0/10	0/10
<u>Marmosa cinerea</u>	6	0/5	0/6
<u>M. murina</u>	3	0/2	0/3
<u>M. parvidens</u>	3	-	0/3
<u>Metachirus nudicaudatus</u>	22	0/21	0/22
<u>Didelphis marsupialis</u>	21	0/20	0/21
Chiroptera			
<u>Saccopteryx leptura</u>	1	-	0/1
<u>Pteropteryx macrotis</u>	2	0/1	0/2
<u>Pteronotus parnellii</u>	18	0/15	0/17
<u>P. personatus</u>	2	0/1	0/2
<u>Tonatia bidens</u>	2	0/2	0/2
<u>T. brasiliensis</u>	3	0/2	0/3
<u>T. silvicola</u>	2	0/2	0/2
<u>Phyllostomus discolor</u>	13	0/11	0/13
<u>P. elongatus</u>	5	0/4	0/5
<u>P. hastatus</u>	2	0/2	0/2
<u>P. latifolius</u>	1	0/1	0/1
<u>Trachops cirrhosus</u>	3	0/2	0/3
<u>Glossophaga soricina</u>	12	0/8	0/12
<u>Lionycteris obscura</u>	1	-	0/1
<u>L. spurrellii</u>	3	0/2	0/3
<u>Lonchophylla thomasi</u>	5	0/3	0/5
<u>Carollia brevicauda</u>	360	0/338	0/359
<u>C. castanea</u>	1	0/1	0/1
<u>Rhinophylla fischeriae</u>	19	0/13	0/18
<u>R. pumilio</u>	3	0/3	0/3
<u>Sturnira lilium</u>	76	0/73	0/76
<u>S. tildae</u>	17	0/15	0/17
<u>Uroderma bilobatum</u>	4	0/3	0/4
<u>Vampyrops helleri</u>	8	0/8	0/8
<u>Vampyressa bidens</u>	9	0/8	0/9
<u>Artibeus cinereus</u>	15	0/11	0/15
<u>A. concolor</u>	1	0/1	0/1
<u>A. fuliginosus</u>	7	0/6	0/7
<u>A. jamaicensis</u>	2	0/2	0/2
<u>A. literatus</u>	24	0/24	0/24
<u>A. sp.</u>	10	0/9	0/10
<u>Ametrida centurio</u>	3	0/1	0/3

TABLE 17. Viruses isolated from mammals collected in the Curua-Una study area, Pará, Brazil, 15 June 1977 through 9 February 1978 - Cont.

Species	Total collected	Blood pos/tested	Viscera pos/tested
<u>Desmodus rotundus</u>	8	0/8	0/8
<u>Eptesicus brasiliensis</u>	2	-	0/2
<u>Molossus</u> sp.	2	0/1	0/2
Primates			
<u>Callicebus torquatus</u>	1	0/1	0/1
<u>Alouatta belzebul</u>	5	0/4	0/5
<u>Callithrix argentata</u>	8	0/5	0/7
Edentata			
<u>Tamandua tetradactyla</u>	3	0/2	0/2
<u>Bradypus variegatus</u>	3	0/3	0/3
<u>Choloepus didactylus</u>	1	0/1	0/1
<u>Dasypus novemcinctus</u>	3	0/2	0/3
Rodentia			
<u>Sciurus gilvularis</u>	3	0/3	0/3
<u>Oryzomys bicolor</u>	12	0/12	2/12*
<u>O. capito</u>	38	0/37	0/38
<u>O. concolor</u>	9	1/7*	1/8*
<u>Rhynchomys</u>	1	-	0/1
<u>Rattus rattus</u>	1	0/1	0/1
<u>Agouti paca</u>	1	0/1	0/1
<u>Dasyprocta prymnolopha</u>	2	0/2	0/2
<u>Proechimys guyanensis</u>	48	0/48	0/48
<u>P. longicaudatus</u>	172	0/162	1/161+
<u>Mesomys hispidus</u>	4	0/4	0/3
<u>Dactylopsax dactylinus</u>	1	0/1	0/1
Carnivora			
<u>Nasua nasua</u>	5	0/1	0/5
<u>Felis wiedii</u>	1	0/1	0/1
TOTAL	1,032	937	1,014

* Flexal virus
+ Urucuri virus

TABLE 18. Distribution of hemagglutination inhibiting antibody to Oropouche and Mayaro viruses in birds collected in the Curuá-Una study area, Pará, Brazil, 15 June 1977 through 9 February 1978.

Family	Oropouche pos/tested	Mayaro pos/tested	Isolation/ tested
Tinamidae	0/1	0/1	0/1
Accipitridae	0/1	0/1	0/1
Falconidae	0/1	0/1	0/3
Columbidae	0/5	0/5	0/8
Cuculidae	0/2	0/2	0/2
Caprimulgidae	-	-	0/3
Trochilidae	0/3	0/3	0/5
Trogonidae	0/7	0/7	0/8
Alcedinidae	0/1	0/1	0/1
Momotidae	0/5	0/5	0/5
Galbidae	0/5	0/5	0/5
Bucconidae	0/18	0/18	0/23
Picidae	0/8	0/8	0/8
Dendrocolaptidae	3/130	1/130	0/159
Furnariidae	0/22	0/22	0/23
Formicariidae	35/259	12/259	1*/321
Conopophadidae	0/1	0/1	0/1
Cotingidae	0/12	0/12	0/14
Pipridae	0/41	0/41	0/48
Tyrannidae	0/38	0/38	0/54
Troglodytidae	4/45	2/45	0/52
Turdidae	0/2	0/2	0/3
Sylviidae	0/1	0/1	0/1
Coerebidae	-	-	0/1
Thraupidae	0/6	0/6	0/7
Fringillidae	0/10	0/10	0/18
TOTAL	42/624	15/624	1*/775

* Kwatta virus

TABLE 19. Results of hemagglutination inhibition (HI) tests for antibody to Oropouche and Mayaro viruses among mammals collected from the Curuá-Una study area, Pará, Brazil, 15 June 1977 through 9 February 1978.

Species	Oropouche pos/no. tested	Mayaro pos/no. tested
Marsupialia		
<u>Monodelphis brevicaudata</u>	0/9	1/9
<u>Marmosa cinerea</u>	0/6	0/6
<u>M. murina</u>	0/1	0/1
<u>Metachirus nudicaudatus</u>	0/22	0/22
<u>Didelphis marsupialis</u>	0/20	0/20
Chiroptera		
<u>Pteropteryx macrotis</u>	0/1	0/1
<u>Pteronotus parnellii</u>	0/3	0/3
<u>Tonatia bidens</u>	0/1	0/1
<u>T. silvicola</u>	0/3	0/3
<u>Phyllostomus discolor</u>	0/9	0/9
<u>P. elongatus</u>	0/3	0/3
<u>P. hastatus</u>	0/2	0/2
<u>Glossophaga soricina</u>	0/5	0/5
<u>Lionycteris spurrelli</u>	0/1	0/1
<u>Lonchophylla thomasi</u>	0/2	0/2
<u>Carollia brevicauda</u>	0/295	5/295
<u>C. castanea</u>	0/1	0/1
<u>Rhinophylla fischeriae</u>	0/4	0/4
<u>R. pumilio</u>	0/1	0/1
<u>Sturnira lilium</u>	0/69	0/69
<u>S. tildae</u>	0/14	0/14
<u>Uroderma bilobatum</u>	0/3	0/3
<u>Vampyrops helleri</u>	0/5	0/5
<u>Vampyressa bidens</u>	0/5	0/5
<u>Artibeus cinereus</u>	0/6	0/6
<u>A. concolor</u>	0/1	0/1
<u>A. fuliginosus</u>	0/6	0/6
<u>A. jamaicensis</u>	0/2	0/2
<u>A. literatus</u>	0/24	0/24
<u>A. sp.</u>	0/5	0/5
<u>Desmodus rotundus</u>	0/7	0/7
<u>Molossus sp.</u>	0/1	0/1
Primates		
<u>Callicebus torquatus</u>	1/1	1/1

TABLE 19. Results of hemagglutination inhibition (HI) tests for antibody to Oropouche and Mayaro viruses among mammals collected from the Curuá-Una study area, Pará, Brazil, 15 June 1977 through 9 February 1978. - Cont.

Species	Oropouche pos/no. tested	Mayaro pos/no. tested
<u>Alouatta belzebul</u>	1/4	3/4
<u>Callithrix argentata</u>	0/5	1/5
Edentata		
<u>Tamandua tetradactyla</u>	0/3	0/3
<u>Bradypus variegatus</u>	0/3	0/3
<u>Choloepus didactylus</u>	0/1	0/1
<u>Dasypus novencinctus</u>	0/2	0/2
Rodentia		
<u>Sciurus gilvicularis</u>	0/3	0/3
<u>Oryzomys bicolor</u>	0/6	0/6
<u>O. capito</u>	0/37	0/37
<u>O. concolor</u>	0/4	0/4
<u>Rattus rattus</u>	0/1	0/1
<u>Agouti paca</u>	0/1	0/1
<u>Dasyprocta prymnolopha</u>	1/2	0/2
<u>Proechimys guyannensis</u>	0/43	0/43
<u>P. longicaudatus</u>	0/165	0/165
<u>Mesomys hispidus</u>	0/3	0/3
Carnivora		
<u>Nasua nasua</u>	0/2	0/2
<u>Felis wiedii</u>	0/1	0/1
TOTAL	3/824	3/824

TABLE 20. Mammals collected by trapping in the Curuá-Una study area, Pará, Brazil, 15 June 1977 through 9 February 1978.

Species	Number collected per trapping site				
	1 - 4	5 - 6	7 - 8	9 - 10	Total
Marsupialia	21	6	1	34	62
<u>Monodelphis brevicaudata</u>	2	-	-	8	10
<u>Marmosa cinerea</u>	3	-	-	3	6
<u>M. murina</u>	1	-	-	1	2
<u>M. parvidens</u>	-	1	-	1	2
<u>Metachirus nudicaudatus</u>	7	-	1	14	22
<u>Dedelpis marsupialis</u>	8	5	-	7	20
Rodentia	111	6	1	168	286
<u>Sciurus gilvicularis</u>	-	-	-	2	2
<u>Oryzomys bicolor</u>	11	-	-	1	12
<u>O. capito</u>	7	-	1	29	37
<u>O. concolor</u>	8	-	-	1	9
<u>Rhynchomys</u>	1	-	-	-	1
<u>Rattus rattus</u>	-	-	-	1	1
<u>Proechimys guyanensis</u>	28	1	-	19	48
<u>P. longicaudatus</u>	53	5	-	114	172
<u>Mesomys hispidus</u>	3	-	-	1	4
TOTAL	132	12	2	202	348
Nights trapped	37	11	7	54	109
Trap nights	6,135	2,024	1,200	9,870	19,229
Mammals/1000 trapnites	21.5	5.9	1.7	20.5	18.1

TABLE 21. Bats collected in mist nets in the Curuá-Una study area, Pará, Brazil, 15 June 1977 through 9 February 1978.

Species	Number collected per trapping site				
	1 - 6	7 - 14	15 - 18	19 - 21	Total
<u>Saccopteryx leptura</u>	-	1	-	-	1
<u>Peropteryx macrotis</u>	-	1	-	-	1
<u>Pteronotus parnellii</u>	15	2	1	-	18
<u>P. personata</u>	-	1	-	1	2
<u>Tonatia bidens</u>	-	2	-	-	2
<u>T. brasiliensis</u>	1	-	1	1	3
<u>T. silvicola</u>	-	1	1	-	2
<u>Phyllostomus discolor</u>	1	3	4	5	13
<u>P. elongatus</u>	1	2	1	-	4
<u>P. hastatus</u>	-	2	-	-	2
<u>P. latifolius</u>	1	-	-	-	1
<u>Trachops cirrhosus</u>	-	3	-	-	3
<u>Glossophaga soricina</u>	-	4	3	4	11
<u>Lionycteris spurrelli</u>	-	3	-	-	3
<u>Lonchophylla thomasi</u>	-	4	-	-	4
<u>Carollia brevicauda</u>	46	178	79	57	360
<u>C. castanea</u>	-	1	-	-	1
<u>Rhinophylla fischeriae</u>	6	11	1	1	19
<u>R. pumilio</u>	2	-	-	1	3
<u>Sturnira lilium</u>	3	28	31	14	76
<u>S. tildae</u>	1	11	-	5	17
<u>Uroderma bilobatum</u>	-	-	1	3	4
<u>Vampyrops helleri</u>	-	6	1	1	8
<u>Vampyressa bidens</u>	-	5	4	-	9
<u>Artibeus cinereus</u>	1	11	2	1	15
<u>A. concolor</u>	-	1	-	-	1
<u>A. fuliginosus</u>	-	4	3	-	7
<u>A. jamaicensis</u>	1	1	-	-	2
<u>A. literatus</u>	1	16	2	5	24
<u>A. sp.</u>	-	9	1	-	10
<u>Ametrida centurio</u>	-	1	2	-	3
<u>Desmodus rotundus</u>	1	4	1	2	8
<u>Eptesicus brasiliensis</u>	-	1	-	1	2
Total	81	317	139	102	639
Nights netted	16	22	12	13	63
Net hours	234	378	194	130.5	936.5
Bats/net hour	0.34	0.84	0.72	0.78	0.68

TABLE 22. Birds collected in the Curuá-Una study area, Pará, Brazil, 15 June 1977 through 9 February 1978.

Family	Site A	Site B	Site C	Total
Tinamidae	-	1	-	1
Accipitridae	-	1	-	1
Falconidae	-	2	1	3
Columbidae	5	-	3	8
Cuculidae	-	2	-	2
Caprimulgidae	2	1	-	3
Trochilidae	2	2	1	5
Trogonidae	1	4	3	8
Alcedinidae	1	-	-	1
Momotidae	-	2	3	5
Galbidae	2	1	2	5
Bucconidae	14	6	3	23
Picidae	2	5	1	8
Dendrocolaptidae	30	92	38	160
Furnariidae	10	9	4	23
Formicariidae	119	129	74	322
Conopophadidae	-	1	-	1
Cotingidae	6	7	1	14
Pipridae	28	10	10	48
Tyrannidae	17	21	16	54
Troglodytidae	9	32	11	52
Turdidae	-	1	2	3
Silviidae	-	1	-	1
Coerebidae	1	-	-	1
Thraupidae	1	-	6	7
Fringillidae	1	8	9	18
TOTAL	251	338	188	777
Mornings netted	23	18	18	59
Birds/morning	10.9	18.8	10.4	13.2

years in captivity, its normal life span may be much less. The larger monkey species may live considerably longer in the wild than the marmosets, thus the probability of being exposed to a virus during its lifetime would be greater.

The members of the antbird family (Formicariidae) were the most common birds collected, and had the highest antibody rate to both ORO virus and Mayaro virus of all birds collected. Although this is a very difficult group of birds to keep in captivity, experimental inoculation with ORO virus and Mayaro virus to determine if they can circulate the viruses at a sufficient titer to infect insects feeding on them are warranted, and initial attempts to do this are reported in a following section of this report.

Although the forest vegetation north of the river appears very similar to that south of the river, the mammal and bird collecting results indicate the density of the animals is higher in the northern sites. The hydroelectric plant was completed in 1977 and many construction workers spent their free hours hunting in the surrounding forest. The forest south of the river was the most accessible and consequently the most heavily disturbed.

LITERATURE CITED

Reference:

1. Beard, J. S.: Climax vegetation in Tropical America. Ecology, 25(2): 127-158. 1944.

2. The Cachoeira Porteira study

a. Potential insect vectors.

OBJECTIVE: The objective of the program of study in Cachoeira Porteira is to investigate the ecology of arboviruses in an undisturbed tropical forest. This section deals with entomological investigations made near Cachoeira Porteira, Par , Brazil, with the specific objective of presenting a checklist of potential insect vectors found there.

BACKGROUND: When construction of the Transamazon Highway south of the Amazon River was initiated, a sister plan was also under consideration. This plan called for the development of a second highway parallel to the Amazon River, but to the north. As part of this plan, a base camp was established at Cachoeira Porteira on the eastern bank of the Trombetas River. This camp served to house workwork crews, professional staff, and equipment used in the construction of the highway.

From this base camp an access road was constructed. This road passes almost due north from Cachoeira Porteira for more than 100 km, and cuts through some of the least disturbed tropical forest of the world. No one lived in this area when this road was constructed.

Shortly after completion of the access road, the entire northern highway program was abandoned. As a result, this area has remained almost unspoiled, yet relatively easy access to this undisturbed tropical forests is available. Even now, almost no human activity is evident in this area.

The base camp has been leased by Andrade Gutierrez, a firm which is developing a large bauxite deposit south of Cachoeira Porteira, also on the Trombetas River. They now use the camp as permanent housing for the professional staff of the mine. The road is maintained by the Brazilian government and occasionally the military conducts training exercises along it. No plans have been made public to renew construction of the highway.

DESCRIPTION: Collections have been made at various sites along the access road. Generally field teams construct a camp along the highway, then collect mammals, birds and insects from several localities within a 2 or 3 km radius from the camp. Collections are usually conducted for approximately 15 consecutive days during each trip, and trips are usually made about 3 times per year.

Habitats vary considerably along the highway and it would not be practical to provide a detailed description of collection sites here. General characteristics of the area are predominated by the near total absence of human activity. Aside from the effects of road construction itself, little else has been disturbed. One result of road building has been to slow or stop the flow of some small streams, which has caused the formation of several small impoundments of water. These impoundments flood low-lying areas and kill the vegetation there, resulting in ideal breeding sites for certain species of mosquitoes, especially some Anopheles.

Little or no logging has taken place along the highway, consequently the forest is taller than that seen in most other areas, and the canopies are more distinct. Aside from immediately adjacent to the road, few indicators of secondary growth such as Cecropia are evident in the area.

Insect collections are conducted nightly using man-biting collections, suction traps and Shannon traps.

PROGRESS: Table 23 presents a summary of the number and species of potential insect vectors collected during trips made in September and November-December, 1976 to Cachoeira Porteira. Clearly the vast majority of insects collected were mosquitoes of the genus Culex. Relatively few canopy species are represented in these collections, most likely because no attempt was made to actively sample this habitat. Consequently, those canopy species presented here represents the few individuals which were caught the forest floor while collections were being made. Likewise, diurnal species are also under represented.

The absence of Culicoides and significant numbers of Phlebotomus from this check list probably also represents a failure on the part of the collectors to actively seek these groups, rather than their true absence from this area.

TABLE 23. Summary of numbers and species of potential insect vectors collected from Cachoeira Porteira, municipality of Oriximiná, Pará, Brazil, 1976.

Species	Data and number collected	
	September	Nov-Dec
<u>Aedes hortato</u>	1	-
<u>Aedes serratus</u>	5	1
<u>Coquillettidia</u> sp.	1	-
<u>Coquillettidia albicosta</u>	3	-
<u>Culex</u> spp.	124	895
<u>Culex</u> sp. B 1	8	455
<u>Culex</u> sp. B 8	6	-
<u>Culex</u> sp. B 17	1	-
<u>Culex</u> sp. B 26	23	-
<u>Culex</u> (<u>Aedinus</u>) sp.	4	-
<u>Culex</u> (<u>Culex</u>) sp.	9	25
<u>Culex coronator</u>	2	15
<u>Culex declarator</u>	12	79
<u>Culex</u> (<u>Lut.</u>) <u>bigoti</u>	1	-
<u>Culex</u> (<u>Melanoconion</u>) sp.	9931	2627
<u>Culex</u> (<u>Mel</u>) <u>portesi</u>	1	-
<u>Culex</u> (<u>Mel.</u>) <u>spissipes</u>	5	3
<u>Culex</u> (<u>Mel.</u>) <u>taeniopus</u>	5	1
<u>Culex</u> (<u>Mel.</u>) <u>vomerifer</u>	2	-
<u>Culex</u> (<u>Microculex</u>) sp.	2	-
<u>Haemagogus</u> spp.	50	1
<u>Uranotaenia calosomata</u>	11	-
<u>Uranotaenia towii</u>	10	-
<u>Uranotaenia leucoptera</u>	2	-
<u>Uranotaenia geometrica</u>	18	57
<u>Limatus flavisetosus</u>	30	4
<u>Limatus paraensis</u>	10	-
<u>Limatus pseudomethysticus</u>	13	-
<u>Phoniomyia</u> spp.	1	-
<u>Sabethes</u> (<u>Sab.</u>) <u>amazonicus</u>	6	-
<u>Sabethes</u> (<u>Sab.</u>) <u>belizarioi</u>	14	-
<u>Sabethes</u> (<u>Sab.</u>) <u>quasicyaneus</u>	2	-
<u>Sabethes</u> (<u>Sab.</u>) <u>cyaneus</u>	21	1
<u>Sabethes</u> (<u>Sab.</u>) <u>tarsopus</u>	2	-
<u>Sabethes</u> (<u>Sab.</u>) <u>chloropterus</u>	14	7
<u>Sabethes</u> (<u>Sab.</u>) <u>glaucodaemon</u>	37	-
<u>Trichoprosopon digitatum</u>	1	-
<u>Wyeomyia</u> spp.	70	4
<u>Wyeomyia aporonoma</u>	2	-

TABLE 23. Summary of numbers and species of potential insect vectors collected from Cachoeira Porteira, municipality of Oriximinã, Pará, Brazil, 1976. - Cont.

Species	Data and number collected	
	September	Nov-Dec
<u>Anopheles albimanus</u>	-	5
<u>Anopheles benarrochi</u>	-	9
<u>Anopheles intermedius</u>	2	-
<u>Anopheles nunez-tovari</u>	47	51
<u>Anopheles oswaldoi</u>	7	22
<u>Anopheles triannulatus</u>	14	3
<u>Phlebotomus</u> app.	1	1
TOTAL	10.531	4.266

b. Vertebrate host serology

OBJECTIVES: The objective of this section is to define through a serological survey those feral vertebrate hosts which have been exposed to Oropouche virus.

BACKGROUND: The environs and program of study at Cachoeira Porteira have been presented elsewhere. Briefly, investigations in this area attempt to study the ecology of arboviruses in a habitat essentially devoid of human inhabitants.

DESCRIPTIONS: Three separate trips to Cachoeira Porteira were made during 1977. On each trip, teams were away from Belém a maximum of 30 days, and usually 15 to 20 actual collecting days were managed within this period. During each trip birds, mammals and insects were collected.

Birds were collected using Japanese mist-nets placed along cleared trails in the forest. All nets were placed at ground level, and no attempt was made to sample birds in the canopy. Nets were opened before sunrise, and were closed between 11a.m. and 12 noon. Nets were checked at about 30 minutes intervals, and captured birds were removed and taken to a field station where they were bled from the jugular vein using a heparinized needle and syringe. A portion of the blood taken was diluted immediately in nutrient media and stored in liquid nitrogen until processed in Belém. The remaining blood was allowed to clot and sera was saved to test for the presence of antibody. Surviving birds were leg banded and released.

Mammals were trapped or shot. Like birds, each mammal captured was bled, and an aliquot stored in liquid nitrogen to be tested for the presence of virus, and the remaining blood allowed to clot and the serum saved to test for antibody. Only very small mammals were bled with heparinized syringes.

Sera were tested for the presence of antibody by the standard hemagglutination inhibition (HI) test, as described by Shope¹. Sera were treated with acetone, then tested at 1:20 dilution against 4 hemagglutinating units of different viral antigens. Whenever a serum was inhibited at the 1:20 dilution, it was retested at additional dilutions of 1:40 through 1:320. The strain of Oropouche (ORO) virus used in all tests was Be An 19991 from infected hamster sera.

Most, but not all, sera which were positive by HI tests were confirmed by neutralization tests conducted in microtiter plates using Vero cells.

At one point during the study, several bird sera could not be confirmed by neutralization tests. Close examination of the bleeding techniques used by the field personnel revealed that they were using a highly concentrate solution of heparin to moisten their syringes. To avoid the possibility of non-specific inhibition due to contaminant heparin, all sera were treated with protamine prior to testing².

PROGRESS: A total of 508 bird sera were collected during three separate trips to Cachoeira Porteira in 1977. These sera represent 25 separate families of birds. Formicariidae birds contributed the largest number of individuals, with 194. All bird sera collected were tested by HI for antibody to ORO virus, and all sera were found to be negative. Table 24 presents a list of bird sera collected by family during each of the 3 trips made to Cachoeira Porteira during 1977.

A total of 273 mammal sera were collected during the 3 trips made in 1977. Of these, 74 represented sera from 5 different species of marsupials. All these sera were negative for HI antibody to ORO virus. Rodents contributed 133 sera from 10 different species, and again, all sera were negative for HI antibody to ORO virus.

Thirty-seven sera were collected from primates hunted in Cachoeira Porteira, and of these, 10 (27%) contained HI antibody to ORO virus. All positive sera were confirmed by neutralization tests. Positive species of primates included Alouatta seniculus (2 pos/5 tested), Ateles paniscus (2/3), Cebus apella (3/15), and Chiropotes satanas (3/10). Of the remaining mammals tested, 6 carnivores, 15 ungulates and 8 other, all lacked HI antibody to ORO virus. A summary of the numbers and species of mammals tested for HI antibody to ORO virus is presented in Table 25.

COMMENT: The only vertebrate group tested which had HI antibody to ORO virus was the primates. Among the primates tested, four of the six species collected had individuals which possessed HI antibody. It appears that the endemic vector of ORO virus must share a habitat utilized by primates, most likely the forest canopy. Further investigations are need to define where monkeys are becoming infected, and by what vector.

The very promising high HI antibody prevalence rates to ORO virus among Formicariidae and other families of birds previously reported appears now to be artifactual. With the protamine treatment of bird sera, all sera originally positive for HI antibody to ORO virus were uniformly negative on retesting.

LITERATURE CITED

References:

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2. Holden, P., Muth, D. and Shiner, R. R.: Arbovirus hemagglutinin-inhibition in avian sera: inactivation with protamine sulfate. *Am. J. Epid.* 84: 67.73, 1966.

TABLE 24. Families and numbers of birds tested for hemagglutination inhibiting (HI) antibody to Oropouche virus from Cachoeira Porteira, km 71, municipality of Oriximiná, Pará, Brazil, 1977. All birds lacked HI antibody to Oropouche virus.

Family	Date and number sampled			Total
	March- April	July- August	Nov- Dec	
Alcediridae	-	2	-	2
Bucconidae	-	1	3	4
Coerobidae	-	-	1	1
Columbidae	-	1	4	5
Conopophagidae	2	3	-	5
Cotingidae	-	1	2	3
Cracidae	1	-	-	1
Dendrocolaptidae	16	21	16	53
Falconidae	-	1	1	2
Formicariidae	78	71	45	194
Fringillidae	1	6	2	9
Furnariidae	9	17	12	38
Galbulidae	1	3	-	4
Momotidae	7	3	2	12
Parulidae	3	-	2	5
Picidae	2	1	-	3
Pipridae	35	20	5	60
Ramphastidae	1	-	-	1
Sylvilidae	3	-	-	3
Thraupidae	6	-	-	6
Tyrannidae	12	9	13	34
Trochilidae	1	-	-	1
Troglotididae	3	5	6	14
Turdidae	13	20	7	40
Vireonidae	-	1	-	1
TOTAL	201	186	121	501

TABLE 25. Distribution of hemagglutination inhibiting antibody to Oropouche virus among mammals captured at Cachoeira Porteira, km 71 municipality of Oriximiná, Pará, Brazil, 1977.

Species	March- April	July- August	Nov- Dec	Total
Marsupials				
<u>Didelphis marsupialis</u>	0/2*	0/6	0/6	0/14
<u>Marmosa cinerea</u>	-	-	0/1	0/1
<u>M. murina</u>	0/4	0/3	0/2	0/9
<u>Monodelphis brevicaudata</u>	0/4	0/3	0/10	0/17
<u>Philander opossum</u>	0/9	0/11	0/13	0/33
TOTAL	0/19	0/23	0/32	0/74
Rodents				
<u>Agouti paca</u>	-	0/1	-	0/1
<u>Dasyprocta aguti</u>	-	0/5	0/2	0/7
<u>Hydrochaeris hydrochaeris</u>	-	-	0/1	0/1
<u>Myoprocta acouchi</u>	0/2	-	0/1	0/3
<u>Neacomys spinosus</u>	-	0/3	0/1	1/4
<u>Nectomys squamipes</u>	0/1	0/7	0/1	0/9
<u>Oryzomys bicolor</u>	-	0/1	-	0/1
<u>O. capito</u>	-	0/15	0/7	0/22
<u>Proechimys guyannensis</u>	0/34	0/28	0/21	0/83
<u>Sciurus gilvigulares</u>	0/1	0/1	-	0/2
TOTAL	0/38	0/61	0/34	0/133
Primates				
<u>Alouatta seniculus</u>	2/2	-	0/3	2/5
<u>Ateles belzebul</u>	-	0/3	-	0/3
<u>A. paniscus</u>	1/1	-	1/2	2/3
<u>Cebus apella</u>	-	0/11	3/4	3/15
<u>Chiropotes satanas</u>	-	2/6	1/4	3/10
<u>Pithecia pithecia</u>	-	-	0/1	0/1
TOTAL	3/3	2/20	5/14	10/37
Carnivores				
<u>Eira barbara</u>	0/1	-	-	0/1
<u>Felis concolor</u>	-	0/1	-	0/1
<u>F. pardalis</u>	0/1	-	-	0/1
<u>Nasua nasua</u>	-	0/1	0/2	0/3
TOTAL	0/2	0/2	0/2	0/6

TABLE 25. Distribution of hemagglutination inhibiting antibody to Oropouche virus among mammals captured at Cachoeira Porteira, km 71 municipality of Oriximiná, Pará, Brazil, 1977. - Cont.

Species	March- April	July- August	Nov- Dec	Total
Ungulates				
<u>Mazama americana</u>	0/1	0/1	0/3	0/5
<u>Tapirus terrestris</u>	-	-	0/1	0/1
<u>Tayassa pecari</u>	-	0/5	0/5	0/8
<u>Dicotyles tajacu</u>	-	0/1	-	0/1
TOTAL	0/1	0/7	0/7	0/15
Other mammals				
<u>Dasypus novemcinctus</u>	0/1	-	-	0/1
<u>Bats</u>	-	0/5	0/1	0/6
<u>Tamandua tetradactyla</u>	-	-	0/1	0/1
TOTAL	0/1	0/5	0/2	0/8

* Number pos/number tested.

3. Mojui dos Campos study

OBJECTIVE: The objective in this study was to determine the antibody level to Oropouche virus in the various wild mammals and birds from Mojui dos Campos area.

BACKGROUND: During January and February of 1975 an Oropouche (ORO) virus epidemic occurred in Mojui dos Campos, and the Instituto Evandro Chagas personnel collected wild mammals and birds in the area during February and March 1975. Their results showed an ORO virus HI antibody prevalence rate of 32.5% among Formicariidae at that time. The overall HI antibody prevalence to ORO virus among all birds and mammals tested was 4.9% (34/681) and less than 1% (1/361) respectively.

The USAMRU ecology team collected wild mammals and birds in the Mojui dos Campos area from 16 February 1978 through 10 March 1978. The mammals and birds were trapped, hunted and mist-netted in the same sites as during the 1975 collections. The objective of this collecting program was to determine if a significant change in the antibody prevalence rates to ORO virus had occurred. An equal or higher prevalence rate would suggest continued transmission of ORO virus in the area, while a lower rate would suggest that vertebrates were infected only during the outbreak, and a drop in prevalence rates could be attributed to normal attrition.

DESCRIPTION: Mojui dos Campos is located 22 km south of Santarém, Pará (latitude 2°37'S, longitude 54°42'W). This area is inhabited by many colonists, without title to their lands, who practice slash and burn agriculture. The entire area is consequently secondary scrub with fruit trees and banana plants near the houses. Mango (*Mangifera indica*), piquiá (*Caryocar villosum*) trees and babaçu (*Orbygnia martiana*) palms are very common. Various types of citrus trees and coffee trees are scattered throughout. Manioc and beans are the most common cultivated crops.

The methods of processing the mammal and bird specimens in the field laboratory located in Mojui dos Campos has been described in previous annual reports.

The bird collecting site was located 5.7 km northwest of Mojui dos Campos and south of the road which runs between Mojui dos Campos and the Santarém-Cuiabá road. Birds were collected from before dawn until 11:00 hrs with mist nets 12 m long and 2.6 m in height. Thirty five nets were placed along a trail which ran through thick secondary scrub. The secondary scrub contained mainly woody plants

with some bamboo and other grasses along the edge of the trail. The woody plants ranged from ground level to about four meters tall. Low palms and trees to about 10 meters in height were scattered throughout. It was impossible to walk through the thicket without cutting a trail. The area was about 50 meters from an overgrown fruit orchard described in bat netting areas 1, 2, and 3.

Bats were collected in mist nets set under fruit trees, and across natural flyways such as trails, edges of clearings, etc. The nets were usually tended from dusk until 22:00 hrs.

Bat nets 1, 2 and 3 were set in an overgrown orchard 5.7 km northwest of Mojui dos Campos and south of the road. The orchard consisted of mango, piquiã and coffee trees, and banana plants. The underbrush was quite thick, although it was possible to walk through without cutting a trail.

Bat nets 4, 5 and 6 were set in the back yard of a house 6.4 km northwest of Mojui dos Campos and north of the road. The nets were set under mango, orange and piquiã trees, and banana plants. The yard was cleared of underbrush, and only some grass and many leaves covered the ground.

Small mammals were collected in National live traps (150x150x485mm) and in Rinker live traps (80x80x255 mm) which were set out at 10 m intervals. The traps were baited with corn and banana, and were checked early in the morning. Larger mammals, such as monkeys and sloths, were hunted.

Trapping area one was located 2.9 km northwest of Mojui dos Campos and south of the road. The traps were set on the edge of a manioc field bordered by a secondary scrub thicket. The secondary scrub consisted of woody plants from ground level to 3 m in height. Babaçú palms to 7 m tall were common. It was not possible to walk without cutting a trail.

Trapping area two was located south of the road 1.6 km northwest of Mojui dos Campos. The traps were placed along trails cut in the very thick secondary scrub vegetation which was composed of woody plants and vines from ground level to about 6 m in height, some scattered cecropia (*Cecropia* sp.) trees were taller.

Trapping area three was located south of the road, 1 km northwest of Mojui dos Campos. Traps were set along the edge of an overgrown manioc field, which contained many shrubs to 4 m in height, and in a thick secondary scrub which bordered the field. The secondary scrub was made up of shrubs from 3 to 5 m tall and babaçú palms to about 6 m in height. Trails were cut through the scrub vegetation for the trap lines.

The fourth trapping area was located behind the house which was used as a field laboratory in Mojui dos Campos. The traps were set in a citrus grove in which the fruit trees were 1 to 2 m in height. The ground was covered with grass, and the grove was surrounded by large mango trees.

PROGRESS: During the 3½ weeks of collecting in the Mojui dos Campos area, 170 mammals representing 20 species, and 305 birds of 14 families were trapped, hunted or mist netted (Tables 26 and 27). No viruses were isolated from the 158 whole blood and 166 viscera specimens tested from mammals, nor the 105 whole blood specimens from birds tested. None of the 136 mammal sera tested contained HI antibody to ORO or Mayaro viruses. Nine of the 304 bird sera tested contained HI antibodies to ORO virus (Table 27). The sera from 18.8% (3/16) of the antbirds (Formicariidae, Formicivora grisea), 4.8% (1/21) of the manakins (Pipridae, Manacus manacus) and 14.7% (5/34) of the wrens (Troglodytidae, Thryothorus leucotis - 1, T. coraya - 4) were positive to ORO virus.

Traps were set out for 14 nights in each of the four trapping sites which produced seven marsupials and 64 rodents (Table 28). Zygodontomys lasiurus (35 specimens) and Oxymycteris sp. (11 specimens) were the most commonly trapped mammal. Mist nets were set out three nights in each of the two netting sites and collected 92 bats. Site 4-6, which had more fruit trees and less undergrowth, produced a higher netting success (3.06 bats/net hour) than net site 1-3 (1.76 bats/net hour).

The nets for collecting birds were operated for 11 days and produced 305 birds, of which the tyrant-flycatcher family, Tyrannidae, (75 collected) were the most common (Table 27).

COMMENTS: The results of this serological survey indicate that the ORO virus HI antibody prevalence rates are lower than those detected following the epidemic investigated during 1975. These results suggest that active transmission of ORO virus is no longer occurring in the Mojui dos Campos area.

The secondary scrub vegetation of the Mojui dos Campos area contrasted greatly with the disturbed primary forest vegetation of Curuá-Una. The species make up of the mammals and birds between the two areas differed accordingly. The two species of Proechimys, normally a forest dwelling rodent, were the most common mammals collected in Curuá-Una, while Zygodontomys, the most common rodent in Mojui dos Campos, was not collected in Curuá-Una. The antbirds (Formicariidae) were the most commonly collected in Curuá-Una but one of the least common in Mojui dos Campos, where the tyrant-flycatchers (Tyrannidae) were the most common.

TABLE 26. Total mammals collected and tested for virus from the Mojui dos Campos study area, Pará, Brazil, 16 February through 10 March, 1978.

Species	Total collected	Sera tested	Blood tested	Viscera tested
Marsupialia				
<u>Caluromys philander</u>	3	2	3	3
<u>Monodelphis brevicaudata</u>	2	2	2	2
<u>Metachilus nudicaudatus</u>	1	1	1	1
<u>Marmosa murina</u>	1	1	1	1
Chiroptera				
<u>Tonatia bidens</u>	1	1	1	1
<u>T. brasiliensis</u>	1	0	1	1
<u>Phyllostomus latifolius</u>	2	2	2	2
<u>Glossophaga soricina</u>	2	0	1	2
<u>Carollia brevicauda</u>	37	23	33	36
<u>Rhynophylla fischeriae</u>	1	0	0	1
<u>Sturnira lilium</u>	28	23	26	28
<u>Uroderma bilobatum</u>	6	2	6	6
<u>U. magnirostrum</u>	1	1	1	1
<u>Artibeus cinereus</u>	13	9	11	11
Edentata				
<u>Bradypus variegatus</u>	7	7	7	6
Rodentia				
<u>Zygodontomys lasiurus</u>	35	33	35	35
<u>Oxymycteris sp.</u>	11	11	11	11
<u>Rattus rattus</u>	2	2	2	2
<u>Proechimys guyannensis</u>	13	13	11	13
<u>P. longicaudatus</u>	3	3	3	3
	170	136	158	166

TABLE 27. Birds collected and tested for HI antibody to Oropouche (ORO) virus and processed for virus isolation from the Mojui dos Campos study area, Pará, Brazil, 16 February through 10 March, 1978

Family	Total collected	Sera tested	Blood tested	HI antibody to ORO virus
Tinamidae	1	1	1	
Rallidae	1	1	1	
Columbidae	44	44	44	
Cuculidae	1	1	1	
Caprimulgidae	1	1	1	
Dendrocolaptidae	3	3	3	
Furnariidae	1	1	1	
Formacariidae	16	16	16	3/16 (18.7%)
Pipridae	21	21	21	1/21 (4.7%)
Tyrannidae	75	74	75	
Troglodytidae	34	34	34	5/34 (14.7%)
Virionidae	13	13	13	
Thraupidae	44	44	44	
TOTAL	305	304	305	9/305(2.9%)

TABLE 28. Mammals collected by trapping and by mist netting in the Mojui dos Campos study area, Par , Brazil, 16 February through 10 March, 1978.

Species	Number collected per site			Total
	Trap sites 1-4	Net sites 1-3	Net sites 4-6	
Marsupialia	7			7
<u>Caluromys philander</u>	3			3
<u>Monodelphis brevicaudata</u>	2			2
<u>Marmosa murina</u>	1			1
<u>Metachirus nudicaudatus</u>	1			1
Chiroptera		37	55	92
<u>Tonatia bidens</u>		1		1
<u>T. brasiliensis</u>			1	1
<u>Phyllostomus latifolius</u>			2	2
<u>Glossophaga soricina</u>			2	2
<u>Carollia brevicauda</u>		9	28	37
<u>Rhinophylla fischeri</u>		1		1
<u>Sturnira lilium</u>		14	14	28
<u>Uroderma bilobatum</u>			6	6
<u>U. magnirostrum</u>		1		1
<u>Artibeus cinereus</u>		11	2	13
Rodentia	64			64
<u>Zygodontomys lasiurus</u>	35			35
<u>Oxymycteris</u> sp.	11			11
<u>Rattus rattus</u>	2			2
<u>Proechimys guyannensis</u>	13			13
<u>P. longicaudatus</u>	3			3
TOTAL	71	37	55	163
Trap nights	1,680			
Net hours		21	18	
Mammals/1000 trap nights	42.3			
Bats/net hour		1.76	3.06	

4. Experimental infections of vertebrates

a. Mammals

OBJECTIVE: These studies are an attempt to define those vertebrate hosts which are capable of producing a substantial viremia following infection, and thus could potentially act as amplifying hosts of Oropouche virus. The section deals with experimental infections of mammals.

BACKGROUND: Mammals collected from localities throughout much of the state of Pará have been tested for the presence of antibodies to Oropouche (ORO) virus. While several thousand individuals have been tested, antibody has only been detected consistently among primates. Primates may serve as the principal vertebrate host of ORO virus in nature, but the sparse abundance of primates and their low reproductive potential indicate that this is a questionable hypothesis, and additional information is required before a conclusion can be drawn on this theory.

Since virtually no rodents have been found to contain antibody to ORO virus, one might conclude that rodents are not involved. One would expect, however, that even if rodents are not actively involved in the maintenance of this virus, an occasional individual would have become infected. Consequently, before they can be discounted completely, it is essential to document that if infected, rodents would produce a normal immune response. This is to insure that the laboratory techniques currently in use, hemagglutination inhibition and neutralization tests, would detect previously infected individuals and that these negative results are in fact a true measurement of exposure.

DESCRIPTION: The rodents most frequently collected in our studies have been members of the genus *Proechimys*. Consequently this group was selected as the initial hosts for experimental infections. Nine *Proechimys* rodents were collected from study sites in Curuá Una, Pará, Brazil and transported live to Belém for experimental infection in the laboratory. Each individual was bled prior to exposure and tested by HI for pre-existing antibody to ORO virus. They were then inoculated subcutaneously with $5.5 \log_{10} \text{TCID}_{50}/0.02 \text{ ml}$ of ORO virus. Rodents were bled daily for seven days post infection, and their whole blood diluted 1:10 in bovine plasma albumin diluent, then frozen at -70°C pending assay for virus. On days 7, 14 and 29 post inoculation each animal was bled for serology as well. Sera were tested by HI and NT using Vero cells grown in microtiter plates. Attempts to isolate virus from

potentially viremic hosts were made by inoculating 0.1 ml of diluted whole blood into duplicate tubes of Vero cells. Cells were observed for 8 days for evidence of CPE.

PROGRESS: No viremia was detected in any of the Proechimys experimentally infected with ORO virus. In addition, even though a relatively high titered inoculum was used, no animal produce HI or N antibody to ORO virus.

COMMENT: These results suggest that Proechimys rodents are refractory to infection with ORO virus. Since data is only available from this single attempt to infect rodents, it is essential that these experiments be repeated before any generalizations can be made. These preliminary results do, however, suggest a plausible explanation for our failure to detect antibody to ORO virus among rodents.

b. Birds

OBJECTIVE: These studies are an attempt to define those vertebrate hosts which are capable of producing a substantial viremia following infection, and thus could potentially act as amplifying hosts of Oropouche virus. This section deals with experimental infections of birds.

BACKGROUND: Serological surveys have found high HI antibody prevalence rates to Oropouche (ORO) virus among certain groups of birds, especially members of the family Formicariidae. Such results suggest that these birds are frequently exposed to feeding by the endemic vector, and that they may provide one means of virus amplification, should they produce a significant viremia following infection. An attempt is made in this study to experimentally infect Formicariidae birds and thereby define their potential to serve as vertebrate amplifying hosts of ORO virus.

Birds of the family Formicariidae are quite common in the forested areas of the New World. Several species have been described, and 23 different species were collected from Cachoeira Porteira during recent trips. Most, if not all, species are insectivores. Their family name is derived from their behavior of following army ants as the ants forage, and feeding on the insects that are flushed up. They apparently do not feed on the ants themselves. They are common in both undisturbed forests and dense secondary growth. Their activity periods generally coincide with that of the ants, and they are especially active in the early morning. They frequent the lower scrubs closest to the forest floor, where they often sit motionless awaiting insects. Few species are found in the forest canopy. Their nesting and roosting sites are unknown, but they are most likely in thickets near the forest floor. They are generally small in size and quite delicate, although some of the larger species approach the size of blackbirds. Formicariidae birds do not migrate.

Two authorities were contacted regarding the biology of Formicariidae birds, Dr. Philip S. Humphrey, Director, Museum of Natural History, University of Kansas, Lawrence, Kansas, USA 66045 and Dr. Edwin Willis, Department of Zoology, UNICAMP, Caixa Postal 1170, Campinas, São Paulo, Brazil, 13100. Both were quite cooperative and provided much of the summary of Formicariidae biology discussed above. They were also questioned regarding the possibility of keeping these birds in captivity, and both agreed

that this is quite difficult, and that the odds of success are very slim.

DESCRIPTION: Formicariidae birds were collected by mist nets from the Utinga forest near Belém. All birds were bled to detect pre-existing HI antibody to ORO virus, then inoculated with approximately 400 plaque forming units of ORO virus. Birds were bled to detect viremia at 24 hour intervals post inoculation and brains, livers and hearts were assayed for virus following death. Whole blood was tested as a 1:10 dilution in bovine plasma albumin; organs as a 10% triturated solution. Blood and organ suspensions were assayed undiluted and at 10^{-2} , 10^{-3} and 10^{-5} . These dilutions were tested at the suggestion of Dr. Pinheiro, who indicated that occasionally ORO virus can only be detected in diluted samples. Blood and organ suspensions were tested in triplicate tubes of Vero cell culture and were observed for cytopathic effect (CPE) for 7 days post-inoculation.

PROGRESS: A total of 14 Formicariidae birds were collected and inoculated with ORO virus. Unfortunately, 10 of these birds died shortly after being infected. Only 4 birds survived at least 24 hours, and all birds died within 48 hours of infection. Cause of death in all cases was due to either the trauma associated with bleeding or failure to feed while in captivity.

Of the 4 birds surviving 24 hours or longer, none demonstrated a detectable viremia following infection, although all lacked pre-existing ORO HI antibody. Assay of livers, brains and hearts also failed to detect ORO virus. These results are summarized in Table 29.

COMMENT: Birds tested in this experiment survived for too short a period to produce meaningful results. Additional work is needed to clarify the role of Formicariidae birds in the maintenance of ORO virus.

This experiment was discontinued due to our inability to maintain the birds in the laboratory. Attempts may be made in the future to construct a large cage to house infected birds in the forest. This may result in longer survival times, but will also present the problem of how to infect potential amplifying hosts without introducing virus into the free-living vector population.

TABLE 29. Summary of virus isolation attempts from Formicariidae birds experimentally infected with approximately 400 plaque forming units of Oropouche virus. Birds were bled at 24 hour intervals post-inoculation, and brain, liver and heart were assayed for virus isolations attempts following death. Material was assayed for virus in Vero cells. All birds tested lacked pre-existing antibody to Oropouche virus.

Species	Virus isolation				
	Hrs post-inoculation		Liver	Heart	Brain
	24 hrs	48 hrs			
<u>Formicarius analis</u>	-	-	-	-	-
<u>Pyriglena leuconota</u>	-	dead	-	-	-
<u>Pyriglena leuconota</u>	-	dead	-	-	-
<u>Thamnophilus aethiops</u>	-	-	-	-	-

II. ECOLOGY OF MAYARO VIRUS

A. Review of the Literature

OBJECTIVE: The objective of this section is to provide a succinct summary of the literature which deals with Mayaro virus. This summary will serve as a preface to the studies reported in the following sections.

BACKGROUND: Mayaro (MAY) virus was first characterized by Casals and Whitman (1957) and found to be closely related to, but distinguishable from Semliki Forest virus. Today it is recognized as an arbovirus of the family Togaviridae, genus Alphavirus (Berge 1975).

Mayaro virus was originally isolated from five humans resident in Southeastern Trinidad and takes its name from Mayaro County, Trinidad, the county in which these people resided. Anderson et al. (1957) described the clinical illness associated with these original five cases, which consisted of fever of several days duration, generalized systemic complaints of headache, chills and general body pain. One patient had a loose bowel movement, and another complained of joint pains and swelling. No rash was reported, and all patients recovered without complications or relapses.

An outbreak of MAY virus which occurred at a rock quarry on the Guamã River in Pará, Brazil was described by Casey and Maroja (1957). Six strains of MAY virus were recovered during investigations of the outbreak, and these strains were also included by Casals and Whitman (1957) in their initial characterization of MAY virus. Clinical illness associated with Guamã River outbreak was very similar to that seen in Trinidad.

A third outbreak was described by Schaeffer et al. (1959). This study reported on epidemic jungle fevers found in a newly formed colony of Okinawan settlers in eastern Bolivia. While several different etiologic agents were probably responsible for this outbreak, only MAY virus was actually isolated. The clinical summary of the single patient from whom MAY virus was isolated is not significantly different from that originally described by Anderson et al. (1957), with the exception that this patient had a mild, generalized maculopapular erythema which appeared on the 6th day of illness and persisted for 2 days. A serological survey of those settlers indicated that 10-15% of the epidemic jungle fevers seen in this settlement could be attributed of MAY virus infection.

Ecological investigations have failed to define the natural cycle of MAY virus. Several species of mosquitoes have been the source

of MAY virus isolations, including Mansonia venezuelensis, Haemagogus spp., Culex sp., Sabethes sp. and Psorophora sp. (Berge 1975). However, no clear association between a vector species and MAY virus has yet been demonstrated. Likewise, no vertebrate host has been suggested as a principal amplifying host for MAY virus, although it was once isolated from an orchard oriole (Icterus spurius) migrating into Louisiana, USA (Calisher et al. 1974).

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II. ECOLOGY OF MAYARO VIRUS

B. Studies on the Epidemic Cycle - the Belterra Outbreak

1. Description of Belterra

OBJECTIVE: The objective of this section is to provide a detailed description of Belterra, Parā, Brazil. This description will then serve as background information for the discussions concerning the epidemiology and epizootology of Mayaro virus in Belterra which follows.

BACKGROUND: In February and March of 1978, several cases of an acute febrile disease were observed in Belterra, and three fatalities were recorded. The virus section of the Institute Evandro Chagas was requested to investigate the apparent outbreak. Members of the USAMRU were then asked by the Institute Evandro Chagas to assist in the investigations.

Investigations were begun in March and two arboviruses were identified as responsible for this outbreak: Yellow Fever (YF) and Mayaro (MAY). All deaths were attributed to YF virus. A discussion of preliminary results of clinical, ecological and epidemiological investigations of these outbreaks is included herein.

DESCRIPTION: Belterra is the name given to a rubber plantation originally founded by the Ford Motor Company of Brazil in 1934. This plantation covers a total of 281,000 ha, of which 7,200 have been used for a rubber plantation. The plantation lies on a plateau about 5 km from the eastern bank of the Tapajōs River in the state of Parā, Brazil. The plateau is 175 m above sea level, and the lowland between the plantation and the river is 75 m above sea level. Belterra is approximately 40 km south of Santarēm, the nearest large city (Figure 12).

The plantation was founded prior to World War II, but rubber production has been hampered due to a persistent fungal disease which has attacked the rubber trees. The plantation has, however, remained open and productive although it changed hands in 1945 and is currently owned and managed by the Brazilian Ministry of Agriculture. During the last 20 years much of the plantation has been allowed to be overgrown by secondary vegetation, consequently the ecology of the area is now a mixture of rubber trees and secondary forest, surrounded by more mature, less disturbed forest.

Belterra lies as a rectangle with its length running east to west. The plantation is divided lengthwise by a road (Road 5) which runs through the center of the plot, and is bordered on the north by

Road 1 and the south by Road 7. The plantation is further divided by Roads 2, 4, 6, 8 and 10 which run north-south. The greatest concentration of housing and administration buildings is found in the north-west corner of the plantation. A small hospital is also located in this area. Additional housing is interspersed throughout the plantation, as shown in Figure 13. Housing is provided by the administration of the plantation for all its employees at minimal cost. Medical care is free to employees. Approximately 50% of the housing districts have electricity and all districts have running water, though not inside the houses. Houses are usually constructed of wood, and aside from the established vilas, nearly all are in close proximity to the forest. Few houses have screening, and mosquito netting is generally not used at night.

Most residents of Belterra are associated with some aspect of the plantation. Many people, both men and women, are employed to collect the latex from the rubber trees and are required to enter the forest almost daily. Latex is generally collected in the morning. Some workers maintain their trails in the afternoon, while others tend to their private gardens. Many families have private gardens away from their houses, and frequently the entire family goes to the garden to work. Hunters frequent both the plantation and the adjacent forests. As a result, many residents of Belterra, representing both sexes and all ages, enter the forests frequently and are thus potentially exposed to infected sylvatic vectors.

The administrators of the plantation conducted a census of Belterra in December of 1977 and found a total of 4083 people resident in the area. A population pyramid showing the age and sex distribution of Belterra as measured by this census is presented in Figure 14, and Table 30 presents the number of individuals seen in each age group. It will be noted that a significant portion of the population is over fifty years of age. This can be explained in part by the fact that employees injured during service for the plantation, or those reaching retirement age, are allowed to remain in housing provided by the plantation and continue to receive free medical care. Consequently there is no pressure for this segment of the population to leave. In addition, much of the population below the age of 41 years old was born in Belterra, consequently, established families are present with whom the older segments of the population may remain. Table 31 presents a summary of the total of residents found in each district of Belterra, and Tables 32 through 37 present a summary of the age structure of each district.

Table 38 presents a summary of the place of birth of 397 persons as questioned in a stratified random sample of 10% of the occupied houses of Belterra made during July, 1978. Clearly the majority of persons currently residing in Belterra were born there. Since the

plantation at Belterra has only been in operation for slightly over 40 years, it seems apparent that the conditions at Belterra have resulted in a very stable population.

Table 39 shows the number of households questioned and the number of people residing in those households, as well as the average number of residents per household. This information was also collected from the 10% stratified random sample of occupied households made during July, 1978.

The climate in Belterra is classified as humid tropical according to the Holdridge life zone classification system. A meteorological station which has been operated by the Instituto Nacional de Meteorologia since 1972 is located in Belterra. The average annual rainfall during the 7 year period was 2,109.5 mm. The normal amount of rainfall changes drastically between the 4 month dry season, August, through November, in which less than 55.5 mm of precipitation falls per month, and the 8 month wet season, December through July, in which the monthly average is more than 100 mm. The average monthly temperature normally differs only slightly throughout the year. The dry season month of October is usually warmest with the 26.2°C average temperature, and July is usually the coolest with an average of 23.8°C. During the dry season the average monthly extreme temperatures range from a low of 20.0°C to a high of 32.0°C. The average monthly extreme temperatures range from a low of 19.4°C to a high of 31.3°C during the wet season. According to Gaussen's formula of plotting rainfall against temperature (20 mm of precipitation equals 10°C), a drought period occurs when the rainfall curve falls below that of the temperature. Using this formula (Figure 15), only the month of October normally has a water deficiency.

The actual rubber plantation is located on a flat plateau which was cleared of small trees and shrubs during the late 1930's. The rubber trees which naturally occurred in the area were left and others were planted. The trees of commercial value were cut for lumber. The remaining vegetation was nearly a monoculture of rubber trees, thereby creating a favorable habitat for parasites and diseases. During the last 20 years invading trees and shrubs have been allowed to grow until the plantation now has a continuous secondary scrub undergrowth, which reaches to approximately 15 m in height and contains some lianas and small vines. The soil is mainly a reddish brown clay-sand with a very thin humus layer. The litter layer, composed of fallen leaves and twigs, is from 1 to 5 cm thick. Trails have been maintained throughout the plantation to permit easy access to the rubber trees. Grapefruit, orange, mango, cupuaçu (*Sterculiaceae*), avocado, cacao and banana trees have been planted, both behind and in front of the houses. The secondary growth vegetation has encroached to the edge of the yards, placing many houses in very close contact with the forest.

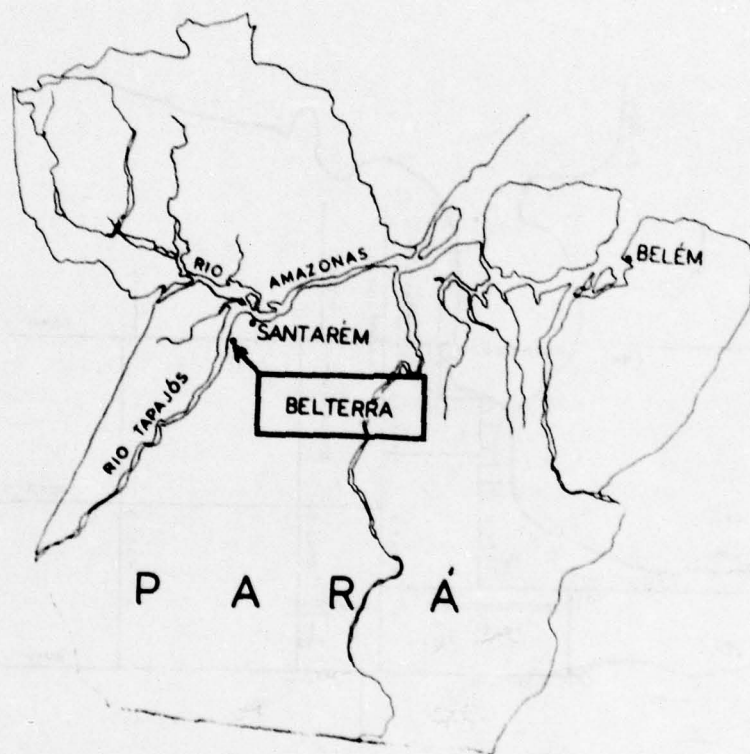


FIGURE 12. Map of the state of Pará, Brazil, showing the location of Belterra.

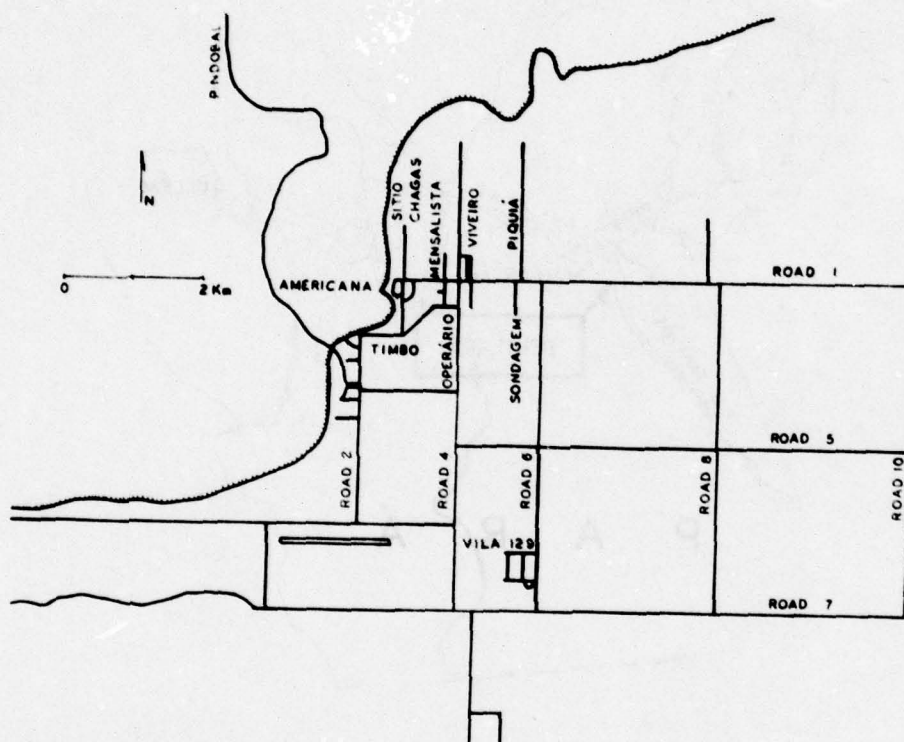


FIGURE 13. Map of Belterra, Pará, Brazil, showing the major roads and names of various residential districts.

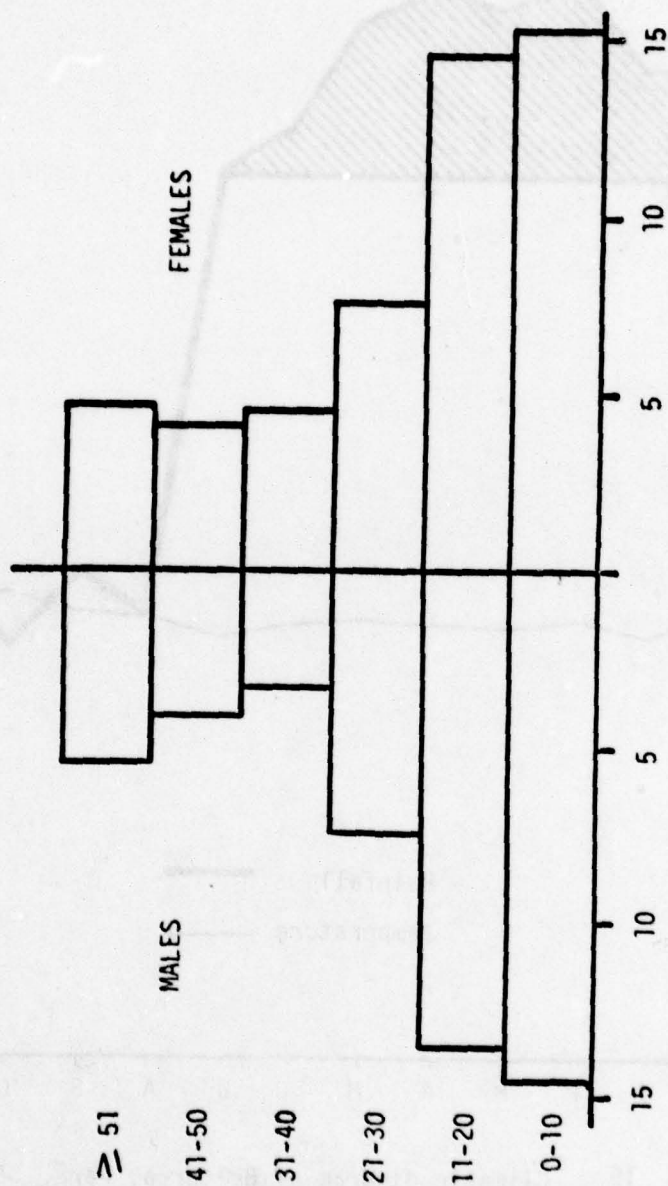


Figure 14. Percent of population in each age group, Belterra, Pará, Brazil, December, 1977.

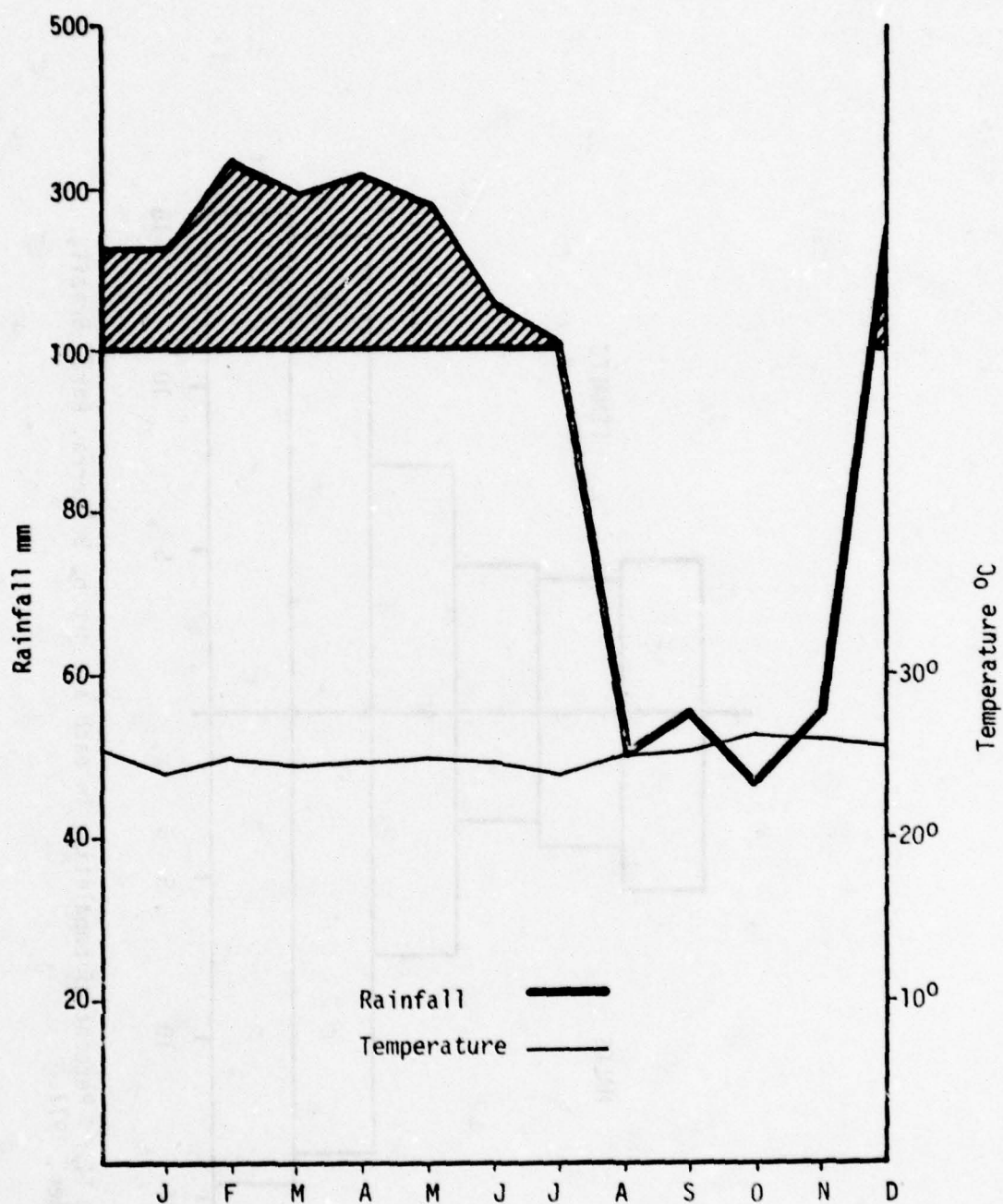


Figure 15 . Climatic diagram of Belterra, Pará. The shaded areas of the curve indicate rainfall over 100 mm per month. This part of the scale has been reduced by 1/10. The monthly temperatures and rainfall were averaged from 6 to 7 years data. (1972-1978)

The lowland forest which occupies the area north and west of Belterra, although subjected to slash and burn agriculture in years past, is less disturbed than the upland forest. The soil there is a brown sandy clay with a very thin humus layer which contains many hair-like roots. The litter layer, from 1 to 5 cm thick, is composed of leaves and twigs. Fallen trees covered by thick moss are common. Woody plants from 1 to 5 m tall are numerous. Shrubs with thin trunks and scattered palms from 3 to 4 m in height make up an open intermediate canopy, and larger trees form a higher canopy approximately 15 m from the ground. The emergents, some exhibiting burn scars, rise to about 30 m in height. No buttressing is evident on the emergents, and only scattered trees have stilt roots. Lianas are common, but not numerous. The vegetation is fairly open, and one can walk in most places without cutting a trail. The inhabitants of Belterra utilize this area for hunting, and thus the trails remain open.

2. Clinical disease in man

OBJECTIVE: The objective of this section is to present a summary of the clinical disease caused by Mayaro virus infection in man as seen in the Belterra outbreak.

BACKGROUND: In February and March of 1978, several cases of an acute febrile disease were observed in Belterra, and three fatalities were recorded. Investigations were begun in March and two arbovirus were identified as responsible for this outbreak: Yellow Fever (YF) and Mayaro (MAY). All deaths were attributed to YF virus. A discussion of preliminary results of clinical investigations of MAY virus are reported here.

DESCRIPTION: Suspect cases of MAY virus infection were actively sought throughout Belterra during investigations of the epidemics. Febrile patients were bled and a clinical history was taken. Blood samples were frozen in liquid nitrogen and returned to Belém where attempts to isolate virus were made by inoculation into suckling mice. In four cases where the clinical history clearly indicated MAY virus infection, but virus was not recovered in suckling mice, attempts were made to isolate virus by directly plaquing whole blood on confluent monolayers of Vero cells grown in 25 cm² plastic flasks.

Selected patients from whom MAY virus was isolated were followed throughout the course of their illness and recovery. These individuals were bled periodically to determine the duration of viremia and onset of detectable antibody. These sera will be used to document the antibody pattern of IgM and IgG production, and samples have been forwarded to the Department of Viruses Diseases, WRAIR, for IgM/IgG level determinations. Detailed case histories were also taken from these patients to determine the duration of clinical signs and symptoms of MAY virus infection.

PROGRESS: Infection with MAY virus was confirmed in 55 (76.4%) of 72 cases seen during the peak of the epidemic. Confirmation was accomplished by isolation of virus from 43 cases, and serologically in an additional 12. Of the 43 cases from whom virus was isolated, 39 isolations were made in suckling mice and 4 were made in Vero cell culture. Virus was identified by hemagglutination inhibition tests using certified reference reagents. The range of ages among patients from whom MAY virus was recovered varied from 2 to 62 years, and both sexes were represented.

Clinical manifestation of disease due to MAY virus included fever,

arthralgia and exanthema. Table 40 presents clinical signs and symptoms associated with MAY virus infection as seen in the 43 patients from whom virus was isolated. Of the clinical symptoms, arthralgia was most predominant and usually manifested in the fingers, hands, feet and ankles, and occasionally effecting the knee or elbow joints. Exanthema was either small maculopapular or micropapular, and was most commonly seen in the thorax, back, upper and lower extremities.

Clinical manifestations persisted for 3-5 days, except exanthema, which could be seen until the 8th or 9th of illness. Arthralgia persisted in some patients, especially older patients, for 2 months or more. No deaths were attributed to infection with MAY virus.

Figure 16 presents a schematic summary of the duration of clinical manifestations of infection with MAY virus, as well as the magnitude and duration of viremia and the onset of detectable hemagglutination inhibiting antibody, as seen in 21 patients bled periodically and from whom MAY virus was isolated. Values for temperature, viremia and antibody are presented as mean values (circles) with ranges superimposed (brackets). Viremia data is presented on a \log_{10} scale, while antibody is presented on a \log_2 scale. Temperatures were measured externally in the axilla, consequently temperatures recorded are somewhat lower than those expected from oral measurements.

COMMENT: In this outbreak, the combined conditions of fever, arthralgia and exanthema were pathognomonic for MAY virus infection. Both fever and arthralgia were seen in all patients from whom virus was isolated, and exanthema was seen in two-thirds of those cases. No other disease was seen which presented itself as a combination of these three conditions.

This is in contrast to the other reported epidemics of MAY virus, where no distinctive clinical syndrome was detected. Arthralgia was recorded in one of five cases in Trinidad described by Anderson et al. (1957), but not among the six patients from whom MAY virus was isolated in the Guamá River, Brazil outbreak reported by Causey and Maroja (1957), or in the single patient from whom MAY virus was isolated in the Bolivian study of Schaeffer et al. (1959). Likewise, exanthema was also reported for a single case, in this instance the Bolivian patient. Clearly MAY virus may present itself in a spectrum of clinical syndromes. The fact that all confirmed patients seen in the Belterra outbreak complained of arthralgia, and two-thirds presented with an exanthema, suggests that the strain of MAY virus which caused this outbreak was especially virulent.

3. Viremia in man

OBJECTIVE: The objective of this section is to quantify the viremic stage of Mayaro (MAY) virus infection in man. The underlying consideration is the question, can man serve as an amplifying host to infect feeding vectors?

BACKGROUND: All patients considered in this section were naturally infected in Belterra during the epidemic which occurred between December 1977 and June, 1978. Previous sections of this report have characterized Belterra and described the clinical syndrome of MAY virus infection in man.

DESCRIPTION: Febrile patients suspected of being infected with MAY virus were bled as described earlier and their blood tested for the presence of virus by inoculation in suckling mice or cell culture. Each sample from which virus was isolated was titrated by directly plaquing 0.1 ml of whole or diluted blood on Vero cells grown in 25 cm² plastic flasks. Cells were incubated for 1 hr at 37°C, then overlaid with nutrient agar. Flasks were stained after 4 or 5 days and plaques counted 24 hrs later. In certain instances when patients presented with symptoms characteristic of MAY virus infection and were bled during the first or second day of illness, but virus was not recovered in suckling mice, the samples were then assayed by directly plaquing on Vero cells as described above.

PROGRESS: Mayaro virus was recovered from a total of 43 patients seen during the Belterra outbreak. Virus was isolated from 96.9% (32 pos/33 tested) patients bled during the first 24 hrs after onset symptoms. Recovery rates then decreased to 82.3% (14/17) on day 2, 22.2% (4/18) on day 3, 6.6% (1/15) on day 4 and 0% (0/13) on day 5. These results are presented in Table 41. The single patient whose blood was negative on day 1 was actually bled at about 12 hrs after the onset of symptoms. This patient was not bled again during the period of typical viremia, and was diagnosed only on the basis of seroconversion. Likewise, the three negative samples drawn on day 2 were also diagnosed solely by seroconversion.

Viremia was detected on days 1, 2, 3 and 4 after the onset of symptoms. Of those bloods tested so far, the maximum titer detected was 9.0×10^3 plaque forming units/0.1 ml whole blood, which was seen on day 1. Titers of viremia were lower on days 2 and 3. Results for day 4 are still pending. A summary of maximum titers detected on each day following the onset of symptoms is presented in Table 42.

COMMENT: Results presented here indicate that the duration of viremia in patients is at most 4 days, and significant titers can be reached in at least 3 of these 4 days. While the quantity of virus needed to infect feeding vectors has not been determined, it appears that man may circulate virus in sufficient quantities to infect some feeding vectors. However, most patients observed during this stage of illness were not continuing their daily activities, and many were bedridden. Consequently, patients would only be expected to be exposed to those vectors found in or near their residencies.

4. Distribution of cases

OBJECTIVES: The objectives of this section are to present the temporal and geographical distribution of cases of Mayaro (MAY) virus as seen in Belterra, and to define the rates of clinically apparent and inapparent cases.

BACKGROUND: A description of the environs and population of Belterra has been presented previously. The census of Belterra made in December, 1977, described earlier, was used as a standard population for all age adjustments.

DESCRIPTION: Information was gathered to construct an epidemic curve for the outbreak in Belterra based on the onset or clinical symptoms characteristic of MAY virus infection. In order to acquire this information, a house-to-house survey of every occupied household was conducted during the last week of May, 1978, and people were questioned for a history of illness compatible with MAY virus infection. Cases with onset in June were estimated from results acquired during a serological survey which was made in July, 1978.

A total of three serological surveys have been made in Belterra. The first was conducted in 1972, and sampled 161 people over the age of 10 years old (y.o.). No information is available as to the sampling frame used or criteria for selection. Consequently, it cannot be assumed that these sera were collected in any type of systematic fashion.

In April, 1978, during the peak of the MAY epidemic, another serological survey was made. This sample contains 327 sera representing all age groups and all residential areas of Belterra. While no formal sampling frame was established, the sample was taken in an attempted random fashion.

The final survey was made during July, 1978, after the end of the MAY outbreak. This was a stratified random sample of 10% of all occupied households in Belterra. The sample was drawn by first numbering all occupied houses by residential area in Belterra, then randomly selecting households. The number of households sampled in each residential area was determined by the percent of households which that area contributed to the total of occupied households. Houses within each residential area were then selected using a table of random numbers. All peoples who occupied the selected houses were bled and questioned for a history of illness compatible with MAY virus. People not at home during the initial visit were

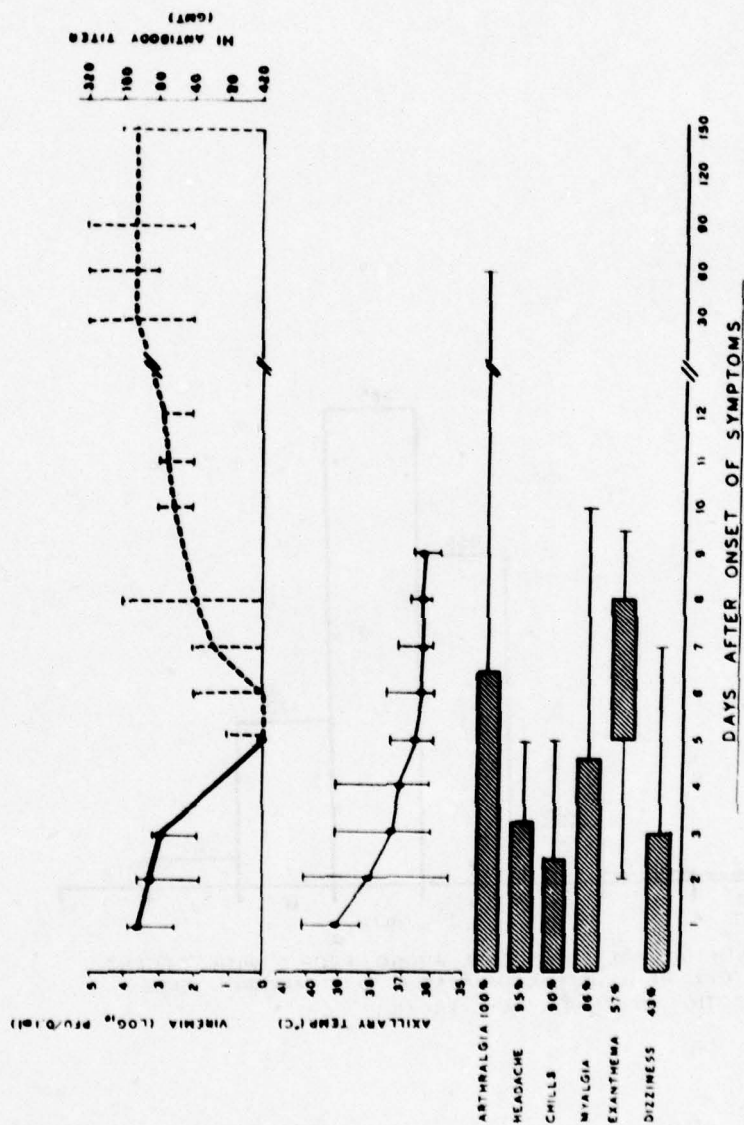


FIGURE 16. Schematic summary of the viremia, temperature and clinical manifestations of Mayaro virus infection as recorded from patients infected during an epidemic in Belterra, Pará, Brazil, 1978.

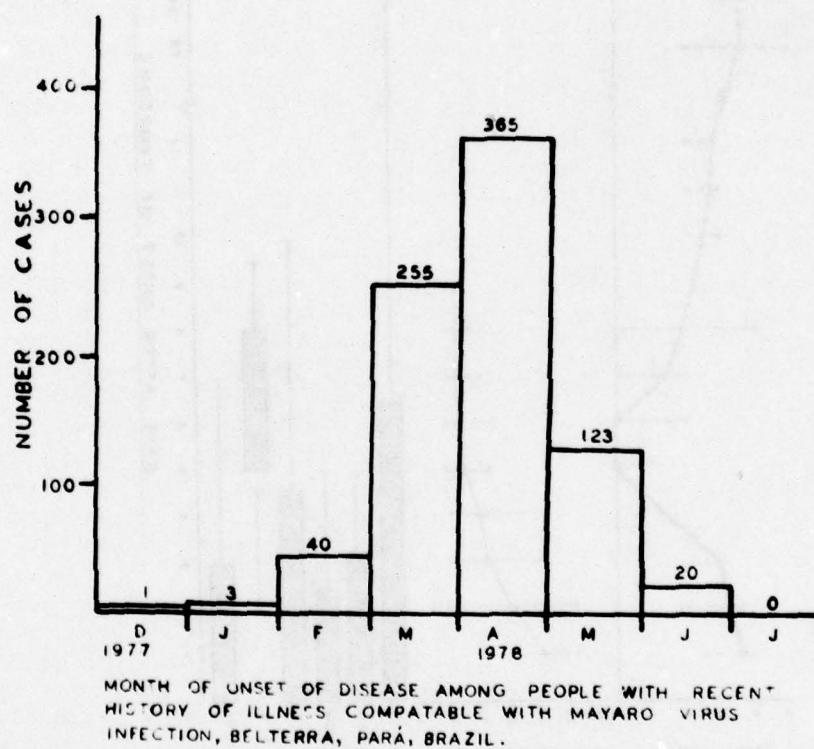


FIGURE 17. Diagram of the epidemic curve for an outbreak of Mayaro virus which occurred in Belterra, Pará, Brazil, 1978.

actively sought, and several return visits were made when necessary to complete the sample.

Sera collected in each survey were tested for the presence of hemagglutination inhibiting (HI) antibody to MAY virus. Samples of both positive and negative results were confirmed by neutralization tests using Vero cell cultures.

PROGRESS: Figure 17 presents a diagram of the epidemic curve for the outbreak of MAY virus in Belterra. Results presented here are based on clinical histories from 3941 people questioned during the last week of May, and for the June cases from the 10% stratified random sample made in July. These results indicate that the epidemic began in December, 1977, reached its peak in April, and the last cases were detected during June, 1978. Active transmission spanned a period of approximately 6 months. The first cases recorded lived at Road 8, and a total of 807 (20.4%) clinical cases were recorded.

Figure 18 presents a summary of the number of people found with HI antibody to MAY virus by age groups. Clearly all ages were exposed to MAY virus infection, and no single age group contributed prominently to the epidemic.

Cases of MAY virus infection were seen in all residential areas of Belterra; however, as shown in Figure 19, the greatest concentration of cases was located in the eastern portions of Belterra. Mayaro virus antibody prevalence rates decreased from east to west, and were lowest in the northwest corner of Belterra, where the population density was greatest, and where housing was farthest from the forest.

In general, the closer that housing was to the forest, the higher the antibody prevalence rates. Characteristic of this association is the higher prevalence rates along Roads 7 and 10, where the population density is low and houses are immediately adjacent to the forests, as compared to Vila 129, also in the southeast part of Belterra, but where houses are clustered together and the forest is not directly adjacent to most houses. This association was not upheld everywhere, though. In Sitio Chagas, a small residential area in the northwest corner of Belterra, houses are sparse and in close contact to the forest; however, this area has a very low prevalence rate of antibody to MAY virus.

The serological survey made in 1972 found a 10.3% age adjusted HI antibody prevalence rate to MAY virus in residents of Belterra above the age of 10. Since no one questioned during the house-to-house survey made during May recalled any illness clinically similar to the present MAY virus syndrome, results of the 1972 survey may serve as an estimate of the pre-existing MAY virus

antibody prevalence rate.

The survey made in April, 1978, at the peak of the epidemic, found an age adjusted HI antibody prevalence rate of about 22%, in which males outnumbered females about 2:1. The July survey, made after the epidemic had subsided, showed 29.7% of the population possessed antibody to MAY virus, and the male to female ratio was nearly equal. Table 43 presents a summary of all 3 serological surveys made in Belterra.

Based on the results of the serological surveys, the house-to-house survey and the census data previously presented, an estimation of clinically apparent and inapparent attack rates can be made. Of the 3941 people questioned in May, 807 (including 20 estimated for June) had a history of clinical illness compatible with MAY virus infection. The serological survey made in July estimated that 29.7% of the population had antibody to MAY virus; however, the survey made in 1972 showed that 10.3% of the population over the age of 10 y.o. had MAY virus antibody at that time. Consequently, a portion of the positive reactions in the July survey represent pre-existing antibody.

Since the 1972 survey only included people over the age of 10 y.o., this must be taken into account when comparing the different surveys. If it is assumed that the 3941 people questioned have the same age distribution as the total census for Belterra, then 70.3%, or 2770 people questioned were over 10 y.o. The July serological survey found a 29.7% age adjusted antibody prevalence rate to MAY virus, or 823 of the 2770 people over 10 y.o. would have had MAY antibody. Of these 2770 people, 10.3%, or 285, would have had pre-existing antibody to MAY virus. Thus 538 (823-285=538) people would be new cases in the 1978 population over the age of 10 y.o.

This assumes that those under 10 y.o. in the 1972 sample had the same pre-existing antibody prevalence rate (10.3%) as did those above 10 y.o. If, however, no one under 10 y.o. had antibody to MAY virus in the 1972 population, then this entire segment would all be susceptible prior to the 1978 outbreak. The 1972 survey was made approximately 5 1/2 years prior to the 1978 survey, consequently 55% of the current 11-20 y.o. age group would be susceptible. A total of 1121 people would be in this age group, and 55% of this equals 617 people. These subtracted from 2770 people over age 10 y.o. leaves 2153 persons at risk of previous antibody, of whom 10.3%, or 222 persons, would have pre-existing antibody.

The previous estimate of 823 serologically positive cases, minus those with pre-existing antibody, should equal the total number of cases of MAY virus which occurred during the outbreak. If those under 10 y.o. in 1972 had the same antibody prevalence rate as the

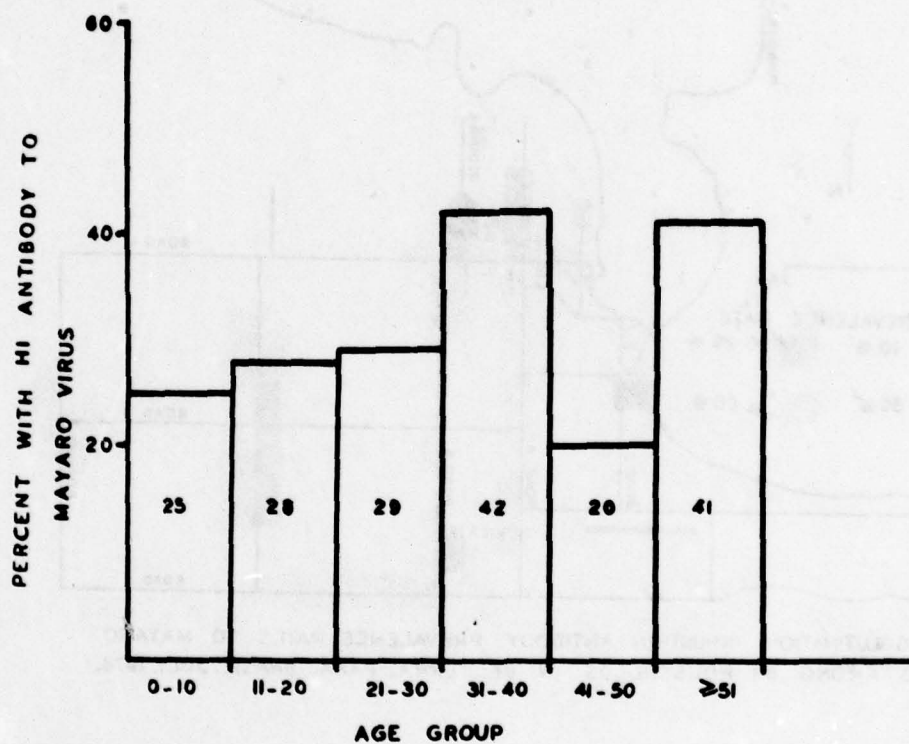
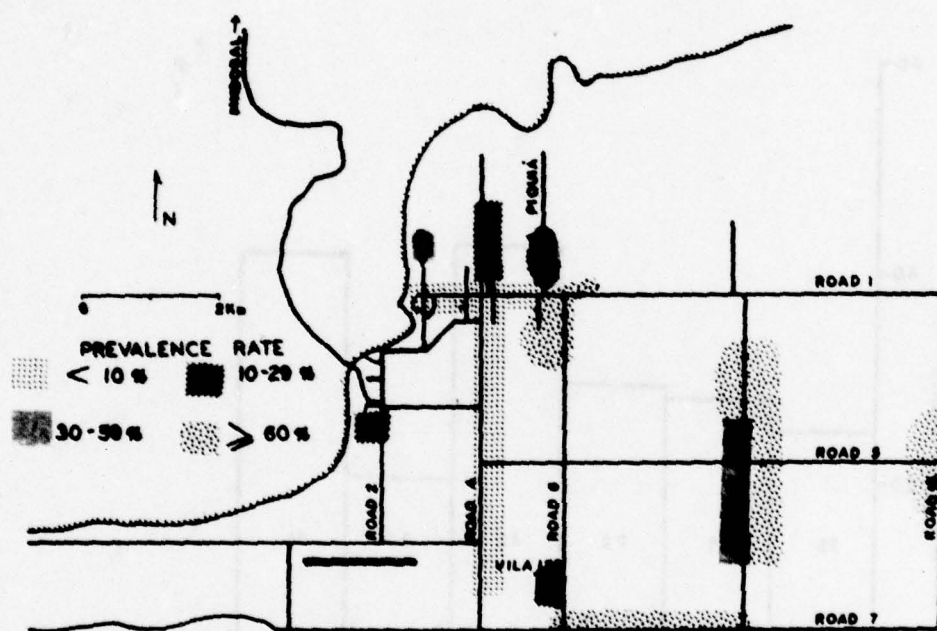


FIGURE 18. Diagrammatic summary of the percent of people found with hemagglutination inhibiting (HI) antibody to Mayaro virus by age as seen among 327 people resident in Belterra, Pará, Brazil, 1978.



HEMAGGLUTINATION INHIBITION ANTIBODY PREVALENCE RATES TO MAYARO VIRUS AMONG 68 HOUSEHOLDS IN BELTERRA, PARÁ, BRASIL, JULY, 1978.

FIGURE 19. Distribution of hemagglutination inhibiting antibody to Mayaro virus among 68 households randomly selected from all residential areas of Belterra, Pará, Brazil, July, 1978.

rest of the population, then 285 would have had pre-existing antibody in 1978 and 538 would represent new cases above age 10 y.o. If those under 10 y.o. in 1972 had no antibody, then 222 would have had pre-existing antibody in 1978, and 601 would represent new cases in the above 10 y.o. age group. Thus the range of new cases above 10 y.o. in 1978 is 538 to 601.

If the 807 clinical histories consistent with MAY virus infection are distributed by age as is the population of Belterra as a whole, then 70.3% of these 807 should be above age 10 y.o., or 567 persons. The estimate for the clinically apparent attack rate is then this value divided by the estimates of new cases just calculated based on serology, or $567/538=100\%$, and $567/601=94.3\%$. Thus, within the above 10 y.o. age groups, at least 94% of the cases of MAY virus which occurred during the Belterra outbreak were clinically apparent. Table 44 presents a summary of these attack rates.

Among the under 10 y.o. age groups, 26% were serologically positive in 1978. A total of 1171 ($2941-2770=1171$) persons are in this age group, consequently 304 (26% of 1171) cases must have occurred. This value, added to the above estimates for the over 10 y.o. ages, represents the new cases of the population as a whole. Thus, $304+538=842$, and $304+601=905$, are estimates of the range of serologically positive new cases of MAY virus. Since 807 represents the total of clinically ill persons, then $807/842=95.8\%$ and $807/905=89.2\%$ are estimates for the clinically apparent attack rate of MAY virus for all ages seen in the Belterra outbreak. Table 45 presents a summary of these attack rates.

In summary, the clinically apparent attack rate for MAY virus in the whole population as studied in Belterra was between 89 and 96%, and in the segment above 10 y.o., the clinically apparent attack rates was between 94 and 100%. Most inapparent cases were then in the under 10 y.o. age group.

A second estimate of the clinical attack rate can be made based on the April serological survey. In this survey, 327 persons were questioned and bled, and 71 were found to have antibody to MAY virus. If 70.3% of the 327 are above the age of 10 y.o., then 230 may have had pre-existing antibody. As measured in the 1972 survey, 10.3% of those above 10 y.o. had antibody to MAY virus, consequently 24 of the 230 would be expected to have pre-existing antibody. The results of questions directed at past clinical illness are summarized in Table 46, and of the 71 antibody positive persons questioned, 23 were asymptomatic. This is very close to the 24 expected to have pre-existing antibody, and these results then also suggest a very high apparent attack rate.

TABLE 30. Population of Belterra, Pará, Brazil as measured by a census taken in December, 1977.

Age group	Males	Females	Total
0 - 5	267	286	553
6 - 10	320	339	659
11 - 20	549	613	1162
21 - 30	296	299	593
31 - 40	141	191	332
41 - 50	165	176	341
> 50	240	201	441
TOTAL	1978	2105	4083

TABLE 31. Census of Belterra, Pará, Brazil by district and sex, December, 1977.

District	Male	Female	Total
Road 1	180	172	352
Road 2	46	44	90
Road 4	247	273	520
Road 6	42	39	81
Road 7	42	34	76
Road 8	446	453	899
Road 10	75	96	171
Sítio Chagas	44	52	96
Vila Americana	20	26	46
Vila Mensalista	34	38	72
Vila Operário	100	132	232
Vila Piquiã	105	82	187
Vila Sondagem	63	75	138
Vila Timbô	61	70	131
Vila Viveiro	272	294	566
Vila 129	201	225	426
TOTAL	1978	2105	4083

TABLE 32. Age and sex distribution of residents of Roads 1, 2 and 4 based on the December, 1977 census of Belterra, Par , Brazil.

Age	Locality					
	Road 1		Road 2		Road 3	
	M	F	M	F	M	F
0 - 5 yrs	23	29	4	4	40	34
6 - 10	33	32	5	5	40	49
11 - 20	55	40	11	14	71	80
21 - 30	20	22	12	7	32	36
31 - 40	14	16	2	1	18	23
41 - 50	14	15	5	8	16	23
> 50	21	18	7	5	30	28
TOTAL	180	172	46	44	247	273
TOTAL	352		90		520	

TABLE 33. Age and sex distribution of residents of Roads 6, 7 and 8 based on the December, 1977 census of Belterra, Pará, Brazil.

Age	Locality					
	Road 1		Road 2		Road 3	
	M	F	M	F	M	F
0 - 5 yrs	6	6	3	1	50	57
6 - 10	7	10	2	2	80	72
11 - 20	13	7	14	11	110	121
21 - 30	2	5	14	10	78	68
31 - 40	6	4	2	6	34	42
41 - 50	-	3	3	2	34	43
> 50	8	4	4	2	60	50
TOTAL	42	39	42	34	446	453
TOTAL	81		76		899	

TABLE 34. Age and sex distribution of residents of Road 10, Sítio Chagas and Vila Americana based on the December, 1977 census of Belterra, Pará, Brazil.

Age	Localities					
	Road 10		Sítio Chagas		Vila Americana	
	M	F	M	F	M	F
0 - 5 yrs	16	14	8	9	2	1
6 - 10	13	15	10	14	3	3
11 - 20	21	29	8	9	7	11
21 - 30	9	12	9	6	3	6
31 - 40	3	9	-	5	2	2
41 - 50	6	8	4	3	3	2
> 50	7	9	5	6	-	1
TOTAL	75	96	44	52	20	26
TOTAL	171		96		46	

TABLE 35. Age and sex distribution of residents of Vila Mensalista, Vila Operária and Vila Piquiã based on the December, 1977 census of Belém, Pará, Brazil.

Age	Localities					
	Vila Mensalista		Vila Operário		Vila Piquiã	
	M	F	M	F	M	F
0 - 5 yrs	3	3	15	16	13	17
6 - 10	2	5	22	31	14	13
11 - 20	7	12	28	35	28	23
21 - 30	1	3	11	17	24	12
31 - 40	8	6	4	17	9	2
41 - 50	5	6	11	7	10	6
> 50	8	3	9	9	7	9
TOTAL	34	38	100	132	105	82
TOTAL	72		232		187	

TABLE 36. Age and sex distribution of residents of Vila Sondagem, Vila Timbõ and Vila Viveiro based on the December 1977 census of Belterra, Pará, Brazil.

Age	Localities					
	Vila Sondagem		Vila Timbõ		Vila Viveiro	
	M	F	M	F	M	F
0 - 5 yrs	8	9	5	7	38	40
6 - 10	9	11	7	8	53	40
11 - 20	20	21	24	30	74	107
21 - 30	5	9	8	7	40	44
31 - 40	7	8	2	7	19	23
41 - 50	6	7	7	4	24	18
> 50	8	10	8	7	24	22
TOTAL	63	75	61	70	272	294
TOTAL	138		131		566	

TABLE 37. Age and sex distribution of residents of Vila 129 based on the December, 1977 census of Belterra, Pará, Brazil.

Age	Vila 129	
	M	F
0 - 5 yrs	33	39
6 - 10	20	29
11 - 20	58	62
21 - 30	28	35
31 - 40	11	20
41 - 50	17	21
> 50	34	18
TOTAL	201	225
TOTAL	426	

TABLE 38. Place of birth of full and part time residents of Belterra by age group as sampled by a stratified random sample of 10% of the occupied houses in Belterra, Pará, Brazil, July, 1978.

Age group	Place of birth		Total
	Belterra	Elsewhere	
0 - 10	103 (77%)	30 (23%)	133
11 - 20	82 (71%)	34 (29%)	116
21 - 30	30 (67%)	15 (33%)	45
31 - 40	15 (50%)	15 (50%)	30
41 - 50	3 (11%)	24 (89%)	27
> 50	1 (2%)	45 (98%)	46
	234 (59%)	163 (41%)	397

TABLE 39. Number of households sampled and the number of full time residents per district and average number of residents per household by district in Belterra, Pará, Brazil based on a 10% stratified random sample of occupied household.

District	# Households	# Residents	X
Road 1	6	34	5.7
Road 2	1	7	7.0
Road 4	9	36	4.0
Road 6	2	12	6.0
Road 7	1	7	7.0
Road 8	16	80	5.0
Road 10	3	13	4.3
Sítio Chagas	2	4	2.0
Vila Americana	1	3	3.0
Vila Mensalista	3	11	3.7
Vila Operário	3	19	6.3
Vila Piquiã	4	19	4.8
Vila Sondagem	2	7	3.5
Vila Timbô	2	12	6.0
Vila Viveiro	8	53	6.6
Vila 129	7	47	6.7
TOTAL	70	364	5.2

TABLE 40. Clinical signs and symptoms among 43 patients from whom Mayaro virus was isolated during the epidemic in Belterra, Pará, Brazil, 1978.

Signs or symptoms	%
Fever	100
Arthralgia	100
Headache	86.0
Chills	81.3
Myalgia	74.4
Exanthema	66.6
Enlarged lymph nodes	52.6
Dizziness	41.8
Eye pain	37.8
Nausea	34.8
Articular edema	23.0
Vomiting	20.9
Photophobia	6.9
Diarrhea	4.6
Conjunctival congestion	2.3

TABLE 41. Number of bloods tested and number of Mayaro virus isolations made by days after onset of clinical symptoms as sampled during an outbreak in Belterra, Pará, Brazil, 1978.

Days of illness	Number of isolations	Number tested	% positive
1	32	33	96.9
2	14	17	82.3
3	4	18	22.2
4	1	15	6.6
5	0	13	0

TABLE 42. Number of bloods titrated for Mayaro virus and the maximum titers found by days after onset of symptoms as sampled during an outbreak in Belterra, Pará, Brazil, 1978.

Days of illness	Maximum titer*	Number tested
1	9.0×10^3	14
2	6.0×10^3	4
3	2.2×10^3	2
4	none tested	0

* Plaque forming units/0.1 ml on Vero cells.

TABLE 43. Summary of HI antibody prevalence rates to Mayaro virus among humans residing in Belterra, Para, Brazil prior to, during and after an epidemic of Mayaro virus disease. All rates are age adjusted to the 1977 census of Belterra.

Date	Sample size	Mayaro aby prevalence rate		
		Males	Females	Total
Nov-Dec 1972	164*	10.8%	3.2%	10.3%
April 1978	327+	32.0%	15.3%	22.3%
July 1978	365+	32.2%	26.1%	29.7%

* Included only above age 10 yrs.; rates based on these denominators.

+ All ages included.

TABLE 44. Maximum and minimum clinical attack rates of Mayaro virus infection among persons over 10 y.o. in Belterra, Pará, Brazil, 1978.

Assumed prevalence of antibody in < 10 y.o. in 1972	Estimated seroconversions in 1978	Actual clinical incidence in 1978	Resulting clinical attack rate
10.3%	538	567	100%
0 %	601	567	94.3%

TABLE 45. Maximum and minimum clinical attack rates of Mayaro virus infection among all persons in Belterra, Pará, Brazil, 1978.

Assumed prevalence of antibody in < 10 y.o. in 1972	Estimated seroconversions in 1978	Actual clinical incidence in 1978	Resulting clinical attack rate
10.3%	842	807	95.8%
0 %	905	807	89.2%

TABLE 46 Clinical manifestations among 71 persons with hemagglutination inhibiting antibody to Mayaro virus as found in a sample of 327 persons interviewed and bled during April, 1978 in Belterra, Pará, Brazil.

Clinical manifestations	No. of persons
Fever and arthralgia with or without rash	40
Fever and rash	4
Fever	4
Asymptomatic	23

5. Vectors in the epidemic cycle

OBJECTIVE: The entomological program was designed to systematically sample hematophagous insects feeding on man by multiple sampling techniques in an effort to identify the principal epidemic vectors of Mayaro (MAY) virus. Additional objectives of the entomological program were:

- a. to identify and quantify the hematophagous insects feeding on man in both the peridomiciliary and working sylvatic environment.
- b. to establish systematic collecting programs and thereby define the temporal and spatial distribution of MAY virus vectors.
- c. to define the geographical distribution and ecological association of MAY virus vector(s).
- d. to identify the epidemic vector(s) of Yellow Fever (YF) virus and, if possible, characterize it as above as well.

BACKGROUND: Simultaneous epidemics of MAY and YF viruses occurred in Belterra, Pará, Brazil. Both epidemics began in December, 1977. The YF virus epidemic was halted by a vaccination campaign which began in mid-April, and the last human case was seen at the end of April. The MAY virus epidemic ended in June, with the last human case seen at that time. A description of the environs of Belterra has been presented previously, as has a summary of the clinical manifestations of MAY virus infection in man. This section deals with investigations of potential insect vectors of both YF and MAY viruses in the Belterra epidemics.

DESCRIPTION: A field entomological surveillance program was initiated in April, 1978 to assist with an epidemiological study of an ongoing epidemic of MAY and YF viruses. Several habitats were sampled to determine the ecological associations of potential insect vectors in Belterra. Those habitats can be considered under two separate headings, the peridomiciliary habitat and the sylvatic habitat.

Peridomiciliary Insect Survey: From the initial epidemiological survey information on current cases of MAY and YF viruses within the Belterra rubber plantation, it was apparent that all members of the family unit were at risk of infection, and that both diseases

were widespread and not limited to a single definable ecological or geographical area. Therefore, it was felt that the peridomestic environment should be methodically sampled throughout Belterra. Peridomestic surveys were conducted primarily during the day, with limited sampling at night. In each area, paired habitats were chosen to be sampled. One site was chosen within 10 to 20 meters of the house, and the other approximately 50 to 100 meters away in the forests. A team of two collectors was located at each site.

"Peridomiciliary" captures were defined as those collections conducted within 10 to 20 meters of the house, normally to the rear of the house within the confines of backyard. "Peridomiciliary-forest" collections were defined as those collections conducted between 50 to 100 meters from the rear of the house, thus generally penetrating into the leading edge of the denser growth of the rubber tree plantation. Ecologically these two habitats shared some similar features, for example, rubber trees were present in both habitats. However, fewer rubber trees were observed within 20-30 meters of the houses and most active households which bordered on the plantation had cleared the lower secondary forest growth near their houses. Banana and mango trees were generally associated with most of the households, and were not present in the forests. "Domiciliary" captures were defined as those collections conducted within a house.

Night Time Surveys: Surveillance for nocturnally active hematophagous insects in the domiciliary and peridomiciliary environments was accomplished by utilizing two survey techniques: man-biting captures and CDC light traps.

Domiciliary surveys were conducted periodically from 18:00-24:00 hrs or from 21:00 to 03:00 hrs by 2 man capture teams. Landing-counts were recorded for 50 minutes per hour capture time. In each collection area, 3 to 4 house were surveyed simultaneously by different teams. Normally, night time collections were made in households where cases of MAY virus had been reported.

Nocturnal peridomiciliary surveys were accomplished by placing CDC light traps near the houses and in adjacent forested areas. When feasible, the light traps were baited with dry ice.

Forest Insect Survey: Sylvatic man-biting insects were surveyed in two distinct ecological habitats: the rubber tree plantation on the plateau and the lowland disturbed primary forest nearer to the Tapajós River. These areas have been described earlier.

In the rubber tree plantation three tree towers were constructed in order to survey the canopy insect fauna. Selection of tree tower locations was based on the following criteria: (1) type of trees available for tower construction; (2) density of tree canopy;

- (3) being representative of the surrounding forest habitat; and
- (4) located within the forest areas where rubber latex was being actively removed.

When possible, concurrent collections in the canopy and at ground level were made, each by two man collection teams. Teams were rotated hourly to prevent fatigue and differences in capture team efficiency. Captures were normally performed from 07:00 to 14:00 and 16:00 to 18:00 hrs each capture day; however, a rigid program was impossible due to uncontrollable environmental and personnel problems.

In the lowland forested area, two tree towers were constructed. One was located on the plain of the lowland area while the second was constructed on the mid-slope down from the rubber plantation plateau. Paired canopy-ground captures were performed at each tree tower from 07:00 to 18:00 hrs with 50 minute capture periods. Ground and canopy teams were rotated hourly as stated previously.

Processing of Insects Specimens: Preliminary processing of insect specimens was conducted at the field station laboratory, with definitive taxonomical determination and virus isolations being performed at the Instituto Evandro Chagas.

In order to prevent excessive mortality of collected insects and possible loss of viral infectivity, insect collections were gathered periodically from all the field sites. Periodic gathering of the material also facilitated the routine processing of the insects. At the field lab, all insects were lightly anesthetized with chloroform, organized in general taxonomic groups and placed in labeled vials. Information pertaining to each collection period was recorded on a field control form. Labeled vials containing the specimens were immediately placed into liquid nitrogen and were transferred to Belém laboratories biweekly. Timely handling and rapid preliminary processing of field specimens collected during the epidemic was a major task and could not have been accomplished without an efficient and dedicated Brazilian staff.

At the Belém laboratories, insects were identified to species and pooled for virus isolations. Sandflies (Psychodidae) and Culicoides (Ceratopogonidae) were grouped into pool sizes of 50, while the pool size for mosquitoes (Culicidae) was 50 individuals or less. Mixed pooling of insects from different collecting areas was performed when the areas were in close proximity, or when only a few specimens of a species were represented. Blood engorged insects were identified; however, these were not pooled for virus isolation attempts.

PROGRESS: The entomological survey at Belterra was initiated on 5 April 1978 and was concluded on 5 May 1978. During this time

approximately 12,000 man biting insects were captured and identified; however, only 9,000 ca. of these were processed for virus isolations due to the presence of undigested blood in many of the insects. Table 47 presents a summary of the species collected and the number of groups tested for virus isolations.

Virus isolations were obtained from 3 species of mosquitoes: Haemagogus janthinomys, Limatus flavisetosus, and Wyeomyia aporonoma; however, only Haemagogus janthinomys yielded isolates of YF and MAY virus. Wyeomyia complex viruses were isolated from Limatus flavisetosus and Wyeomyia aporonoma.

Table 48 and Figure 20 presents a summary of the geographical distribution and ecological association of arbovirus isolates from Haemagogus janthinomys. A review of the data shows that only 2 MAY virus isolates were obtained from H. janthinomys collected from the peridomiliary environs, while 7 strains of MAY virus were isolated from H. janthinomys in the forest further from the residential areas. Seventy seven percent (7/9) of the MAY virus isolates were obtained from Haemagogus captured in the forest canopy. It is also noted that approximately 50% (5/9) of the MAY virus isolates were recovered from the disturbed primary forest lowland bordering the rubber plantation plateau.

Only two strains of YF virus were isolated from 64 pools of Haemagogus tested. One isolate was obtained from collections made in the rubber plantation forest, while the second isolate was from collections made in the lowland forest area. Table 48 indicates that the isolates of MAY and YF viruses appear to be dispersed in a variable pattern among the areas sampled.

Tables 49 and 50 summarize the nocturnal endophilic and exophilic insect species captured in the domiciliary environs. Culex fatigans and Culex coronator were the most abundant man-biting mosquito species collected within the houses, with Culex fatigans being the dominate of the two. Five endophilic species of mosquitoes (Culex, 2 spp; Mansonia, 3 spp.) were recorded during the epidemic.

Light trap collections yielded 7 species of mosquitoes in the peridomiliary environs. Culex coronator was the dominate mosquito species collected. Man-biting and light trap collections indicated that the mosquito populations were low in the peridomiliary environs.

Figures 21, 22, 23 and Table 51 show the diurnal activity for Haemagogus janthinomys in different geographical and ecological areas, which represent paired canopy and ground captures for Haemagogus. Figure 21 indicates that the forested areas near the house exhibited significantly higher numbers of Haemagogus than the

Table 47. Summary list of insects species tested for virus isolation attempts collected during a Yellow Fever and Mayaro epidemic in Belterra, Pará, Brazil, 1978.

List of species	No. of pools	Total No. tested	Virus isolation
<u>Culicoides paraensis</u>	50	2,303	
<u>Limatus durhamii</u>	61	1,472	
<u>Culicoides debilipalpis</u>	24	758	
<u>Haemagogus janthinomys</u>	62	732	(2) Yellow Fever
<u>Limatus flavisetosus</u>	32	720	(9) Mayaro
<u>Phlebotomus spp.</u>	18	574	(1) Myeomyia complex
<u>Myeomyia aporonoma</u>	23	472	(1) Myeomyia complex
<u>Culicoides spp.</u>	11	425	
<u>Culex (C) coronator</u>	22	378	
<u>Trichoprosopon digitatum</u>	14	244	
<u>Sabethes (Sab) belizarioi</u>	9	174	
<u>Myeomyia spp.</u>	11	158	
<u>Sabethes (Sab) glaucodaemon</u>	12	157	
<u>Culex (C) fatigans</u>	1	20	
<u>Culex (M) sp.</u>	4	26	
<u>Culicoides insinuatus</u>	4	123	
<u>Forcipomyia</u>	6	88	
<u>Haemagogus leucocelaneus</u>	1	6	
<u>Psorophora cingulata</u>	6	77	
<u>Sabethes (Sab) chloropterus</u>	2	12	
<u>Sabethes (Sab) cyaneus</u>	1	8	
<u>Sabethes (Sab) quasicyaneus</u>	14	102	
<u>Sabethes (Sab) shannonii</u>	3	13	
<u>Orthopodomyia fascipes</u>	3	69	
<u>Aedes (How) septemstriatus</u>	1	5	
<u>Aedes (How) tulvithorax</u>	1	6	

Total species - 26

Total grupos - 396

Total mosquito- 9,122

/zcm.

Table 48. Geographical distribution of arboviruses isolated from *Haemagogus janthinomys* (Dyar) collected during epidemics of Yellow Fever and Mayaro in Belterra, Para, Brazil, 1978.

Collection site	Habitats sampled				Pools inoculated	Specimens inoculated	Virus isolation	
	Peri-domiciliary	Forested area near house	Forested area				Mayaro	Yellow Fever
			Ground	Canopy				
Vila 129	X	X			3	27	(1)	
Vila Piquiã			X		1	3		
Vila Sondagem	X	X			5	32	(1)	
Vila Coração	X	X			2	18		
Vila Golf	X	X			1	2		
Road 4	X	X			5	16		
Road 8	X	X			4	32		
Road 7	X	X			3	19		
Road 1 Station 1				X	5	132	(2)	(1)
Road 1 Station 1			X		5	66		
Road 6 Station 2				X	1	17		
Road 6 Station 2			X		1	7		
Station 4				X	5	71	(1)	
Station 4			X		2	34		
Station 5				X	14	193	(4)	(1)
Station 5			X		2	20		
Station 4 - 5			X		5	38		
TOTALS					64	727	9	2

/zcm.

Table 49. Summary of peridomestic CDC light trap collections conducted during Yellow Fever and Mayaro epidemics in Belterra, Par , Brazil, 1978.

Species	Forested area near house (50-100 meters)			Peridomestic collections (10-20 meters)		
	No. collected	No. Light-Trap Nights	No. Coll./L-T Night	No. collected	No. Light-Trap Nights	No. Coll./L-T Night
<u>Culex</u>						
<u>coronator</u>	22	8	2.7	20	23	.9
<u>corniger</u>	1	8	.1	5	23	.2
<u>complexo-vomerifer</u>	1	8	.1	2	23	<.1
<u>sp B # 21</u>	1	8	.1	1	23	<.1
<u>Psorophora</u>						
<u>cingulata</u>				1	23	<.1
<u>Uranotaenia</u>						
<u>lowii</u>	1	8	.1	1	23	<.1
<u>callosomata</u>				1	23	<.1
<u>Phlebotomus spp.</u>	9	8	1.1	14	23	.6

/zcm.

Table 50. Summary of nocturnal endophilic insects collected during Yellow Fever and Mayaro epidemics in Belterra, Pará, Brazil, 1972.

Species	Time of Capture							
	19:00-19:50	20:00-20:50	21:00-21:50	22:00-22:50	23:00-23:50	24:00-00:50	01:00-01:50	02:00-02:50
<u>Culex</u>								
<u>fatigans</u>	6/6* (1)**	1/6 (.2)	6/6 (1)	11/6 (.7)	17/10 (1.7)	6/10 (.6)	3/10 (.3)	3/10 (.3)
<u>coronator</u>	3/6 (.5)		2/6 (.3)		3/10 (.3)			2/10 (.2)
<u>Mansonia</u>								
<u>titillans</u>					1/10 (.1)			
<u>amazonensis</u>				1/16 (.06)				
<u>humeralis</u>				1/16 (.06)				
<u>Phlebotomus spp.</u>					4/10 (.4)		3/10 (.3)	4/10 (.4)

* No. of insects collected/No. of 2 man team captures for 50 minutes capture periods.

** No. of insects collected per unit capture (2 man capture for 50 minutes).

/zcm.

Table 5] Journal activity pattern of *Haemagogus janthinomys* (Dyar) in the forested area of a rubber plantation in Belterra during epidemics of Yellow Fever and Mayaro viruses, 1978.

	06:40 07:10	07:20 07:50	08:00 08:30	08:40 09:10	09:20 09:50	10:00 10:30	10:40 11:10	11:20 11:50	12:00 12:30	12:40 13:10	13:20 13:50	15:30 16:00	16:10 16:40	16:50 17:20	17:30 18:00	18:10 18:40	Total
Canopy	0/6*	2/7 (.28)	1/5 (.20)	0/3	1/3 (.33)	4/4 (1.0)	7/7 (1.0)	12/7 (1.71)	13/6 (2.16)	4/5 (.80)	13/5 (2.6)	6/4 (1.5)	24/6 (4.0)	28/7 (4.0)	16/7 (2.3)	3/5 (.60)	134/87 (1.54)
Ground	0/5	0/6	0/7	1/6 (.17)	2/7 (.28)	2/6 (.33)	2/6 (.33)	13/6 (2.2)	11/5 (2.2)	14/4 (3.5)	4/4 (1.0)	3/4 (.75)	8/7 (1.1)	11/7 (1.6)	5/6 (.83)	2/2 (1.0)	78/88 (.87)
Canopy	0/2	0/4				0/4	3/4 (.75)	4/4 (1.0)	2/4 (.50)	1/4 (.25)	1/3 (.33)	0/1	4/3 (1.33)	4/4 (1.0)	3/4 (.75)	0/2	22/43 (.51)
Ground	0/2	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	3/3 (1.0)	0/1	1/4 (.25)	2/4 (.50)	1/4 (.25)	0/2	7/56 (.13)
Canopy	0/3	0/6	0/6	0/6	0/6	0/6	0/6	0/5	0/5	2/5	1/5	0/4	0/4	0/4	0/4	0/4	3/73 (.04)
Ground	0/6	0/12	0/12	0/12	0/12	0/12	0/12	0/10	4/10	0/10	0/10	0/8	0/8	0/8	2/8	0/8	6/79 (.08)

* a/b = Total number of *Haemagogus* spp. collected; b = Number of capture periods.

** () = Number of *Haemagogus* per 2 man capture teams.

085.: Collections were not made between the hours of 14:00 to 15:00 hrs.

/ZCM.

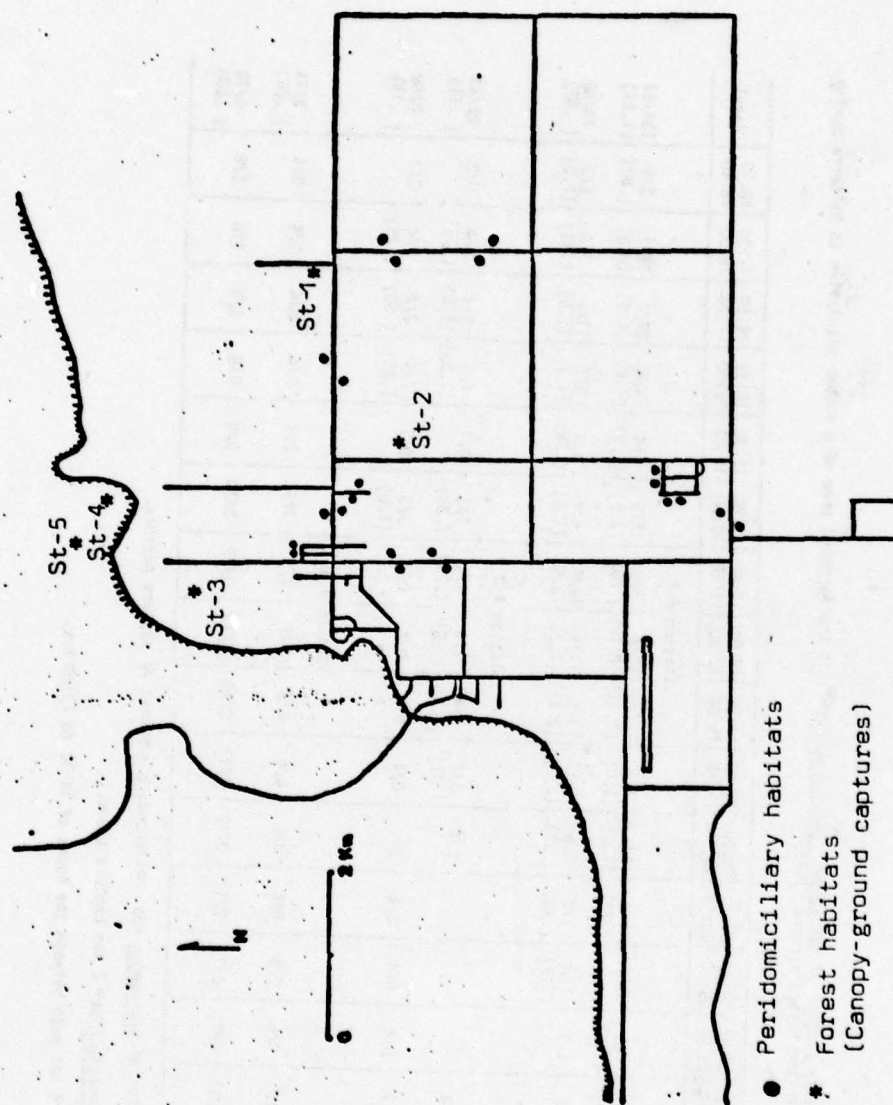


Fig. 20. Spatial distribution of sylvatic and peridomiliary collecting sites in Belterra, Pará, Brazil, 1978.

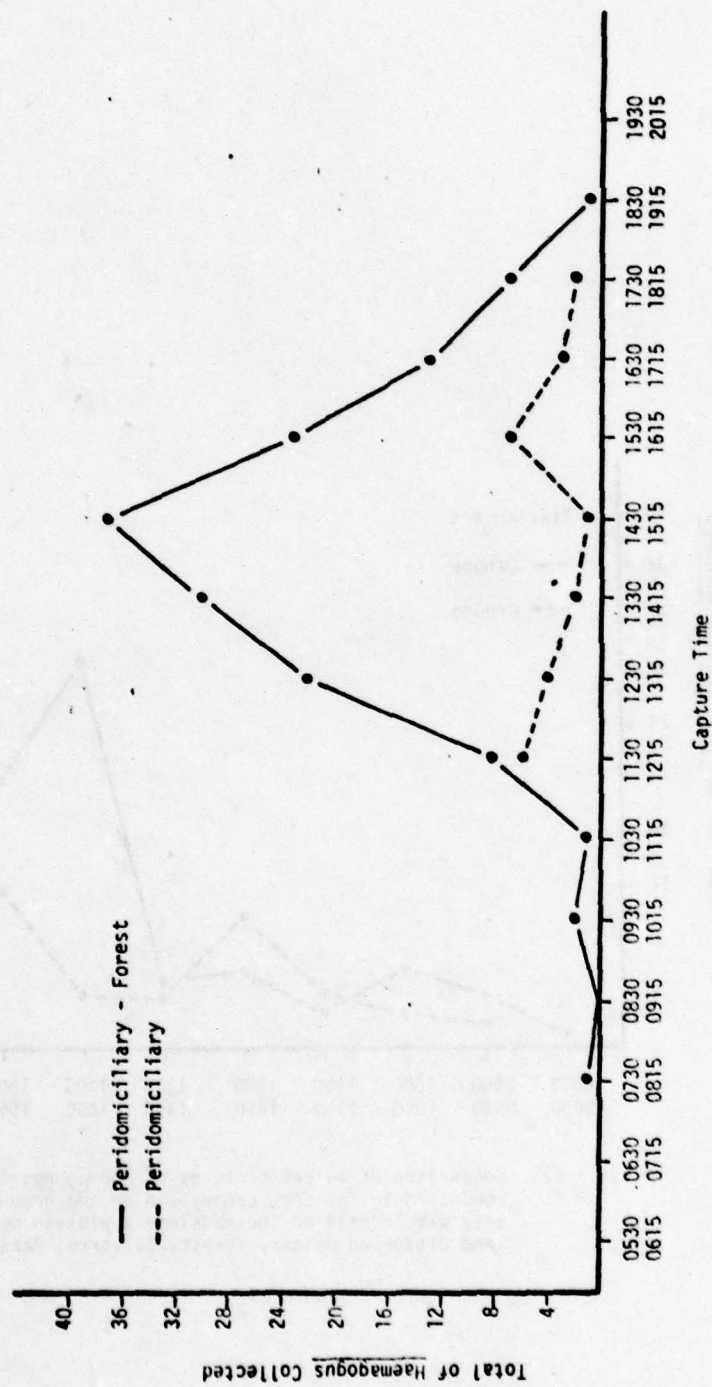


FIG. 21. Paired captures of *Haemagogus janthinomys* (Dyar) in two peridomestic habitats (near the house-yard, 10-20 meters and forested area near the house, 50-100 meters). Captures were conducted in a rubber plantation, Belterra, Pará, Brazil, 1978

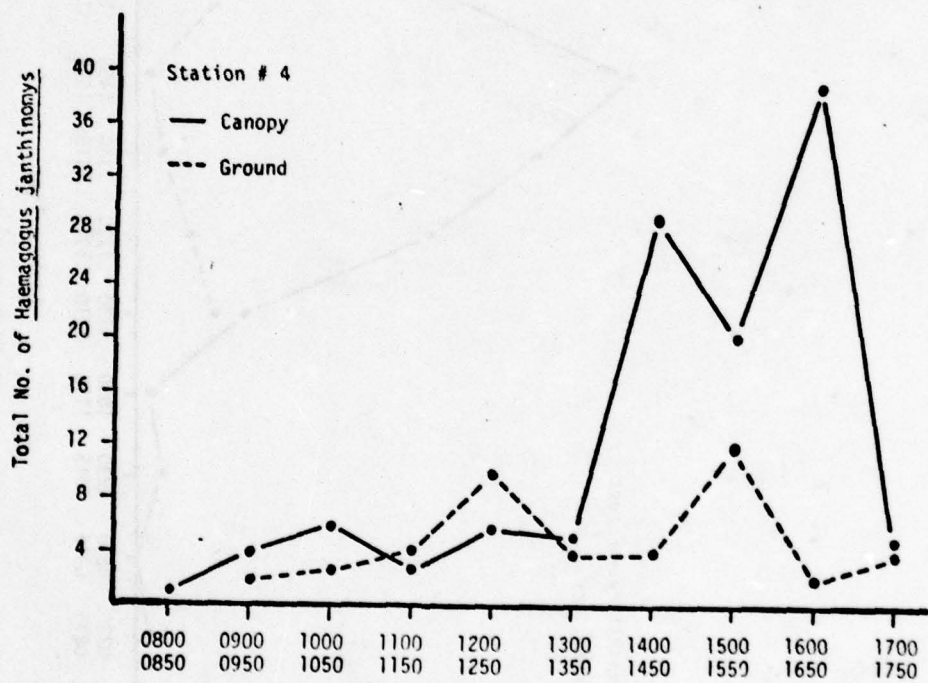


FIG. 22. Comparison of paired captures of *Haemagogus janthinomys* (Dyar) conducted in the tree canopy and on the ground. Collecting site was located on the midslope a plateau descending to a low land disturbed primary forest, Belterra, Pará, Brazil, 1978.

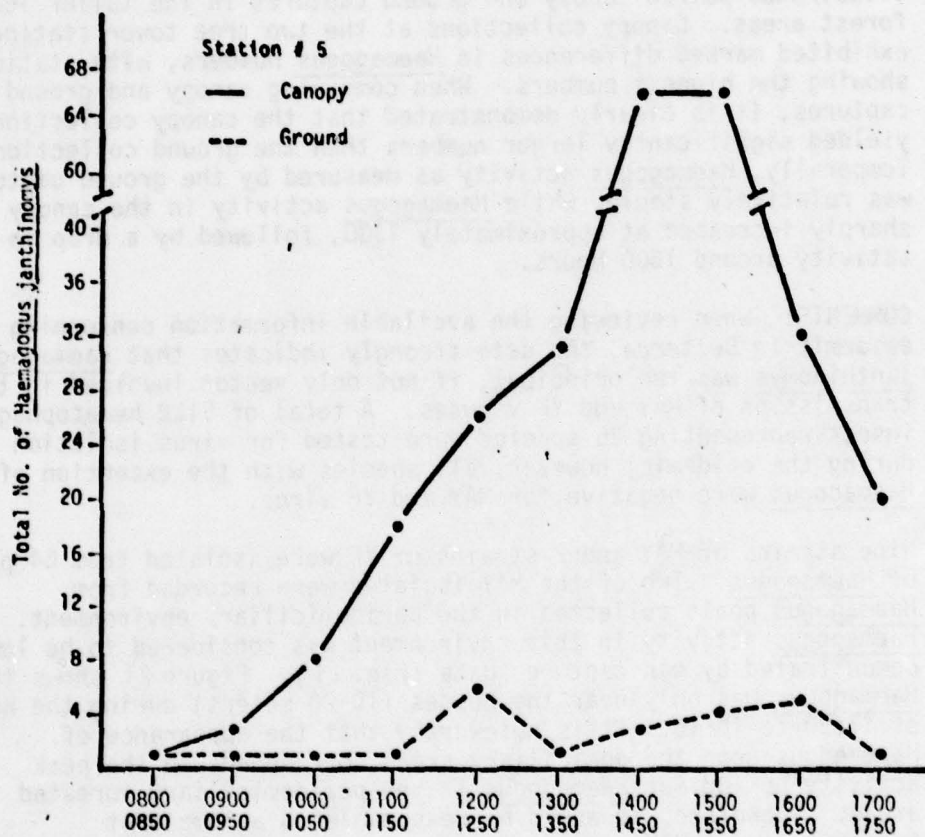


FIG. 23 . Comparison of paired captures of *Haemagogus janthinomys* (Dyar) conducted in the tree canopy, and on the ground in a low land disturbed primary forest habitat, Belterra, Par , Brazil, 1978

environs immediately adjacent to the houses. Haemagogus activity near the residential areas was quite low and relatively stable throughout the times sampled, while Haemagogus activity in the nearby forested areas showed a definite increase from midday to approximately 16:00 hours.

Table 51 summarizes the diurnal activity pattern for the three (3) tree towers located on the rubber plantation plateau. The number of Haemagogus captured from these areas was low; however, the data does show a moderate increase of activity around midday, which continued until approximately 16:00 hours. Figures 22 and 23 illustrated paired canopy and ground captures in the taller lowland forest areas. Canopy collections at the two tree tower stations exhibited marked differences in Haemagogus numbers, with station #5 showing the highest numbers. When comparing canopy and ground captures, it is clearly demonstrated that the canopy collections yielded significantly larger numbers than the ground collection. Temporally, Haemagogus activity as measured by the ground captures was relatively stable, while Haemagogus activity in the canopy sharply increased at approximately 1300, followed by a drop in activity around 1600 hours.

COMMENTS: When reviewing the available information concerning the epidemic in Belterra, the data strongly indicates that Haemagogus janthinomys was the principal, if not only vector involved in the transmission of MAY and YF viruses. A total of 9122 hematophagous insects representing 26 species were tested for virus isolation during the epidemic; however, all species with the exception of Haemagogus were negative for MAY and YF virus.

Nine strains of MAY and 2 strains of YF were isolated from 64 pools of Haemagogus. Two of the MAY isolates were recorded from Haemagogus pools collected in the peridomiliary environment. The Haemagogus activity in this environment was considered to be low as demonstrated by man capture data (Fig. 21). Figure 21 shows that Haemagogus was only near the houses (10-20 meters) during the hours of 11:30 to 18:15. It is noteworthy that the appearance of Haemagogus near the domiciliary areas corresponds to the peak activity period for Haemagogus in the peridomiliary forested areas. Therefore, it would be reasonable to assume that transmission of MAY and YF viruses to man in the peridomiliary environs was most likely to occur in the latter portion of the day (12:00 to 18:00 hrs). Due to the low numbers of Haemagogus occurring near the residential areas, it would appear that most transmission to man was actually occurring in the sylvatic environment where the highest activity of Haemagogus was localized.

Activity of Haemagogus at ground level in the sylvatic environment began at approximately 09:00 hrs and continued until 18:00 hrs, with a moderate increase in this activity occurring during the

latter part of the day. Therefore, any human activity within the sylvatic habitat would be exposed to active Haemagogus for most of the day, with a moderate increase in exposure during the latter part of the day.

The vertical distribution of MAY and YF viruses shows that 7 MAY strains and 2 YF strains were recovered from Haemagogus inhabiting the forest canopy, while two MAY isolates were made from mosquitoes collected at ground level. These results suggest that an arboreal host, perhaps primates, might be involved in the epizootic cycle of MAY virus.

6. Vertebrate hosts in the epidemic cycle

a. Results of a serological survey

OBJECTIVE: The objective of this section is to identify by a serological survey those vertebrate hosts which have been previously exposed to Mayaro (MAY) virus. Species which show a substantial antibody prevalence rate to MAY virus will be tested further to determine if, when infected, they produce a viremia of sufficient titer to infect feeding vectors.

BACKGROUND: Simultaneous outbreaks of MAY and Yellow Fever (YF) viruses occurred in Belterra during 1978. Yellow Fever virus activity was apparently halted following a vaccination campaign conducted during April. The last human cases of MAY virus infection were seen in June.

DESCRIPTION: Birds and small mammals were collected from various sylvatic areas throughout Belterra and the adjacent forests. Birds were collected in Japanese mist nets placed along cleared trails in the forests as described earlier. Trapping sites 1-3, bat netting sites 1-4 and bird netting sites A, B, C and F were in the overgrown rubber plantation described earlier (Figure 24). Trapping site 4 and bird netting site D were in the lowland forest which also has been described. Trapping site 5, bat netting site 5 and bird netting site E were west of the plantation in a tall upland forest with a 20 m canopy formed by trees and palms. The numerous emergents have little or no buttressing and were up to 35 m tall. Trapping site 6 and bat netting site 6 were in an orange grove overgrown with thick grass and woody plants to 2 m in height. Trapping site 7 was in an overgrown field with tall grass and shrubs to 2 m tall. The field was adjacent to a well maintained experimental citrus grove. Trapping site 8 was in a secondary forest with many palms and trees to 15 m in height, and emergents 25 m tall.

Mammals were live trapped or hunted. All animals collected were bled, with whole blood taken to be processed for virus isolation, and sera tested for antibody to various viruses by HI tests. Birds were generally bled with a syringe previously moistened with a diluted heparin solution. Mammals were bled without heparin. All blood samples were tested for virus by intracerebral inoculation into suckling mice. Viruses isolated were identified by hemagglutination tests. Sera were tested for the presence of antibody by standard HI tests as described previously. Results of many, but not all, primate sera were confirmed by neutralization

tests using Vero cells grown in microplates.

PROGRESS: A total of 754 birds were collected from Belterra and adjacent areas during the period 15 March through 21 July 1978. The mist nets collected 723 birds of 20 families during 53 mornings of netting (Table 52). Blood samples were also taken from three domestic ducks and 28 chickens belonging to the local inhabitants. The forest sites produced twice the number of birds per netting period as the plantation. The families of Formicariidae and Pipridae were the most commonly collected birds.

From 15 March through 25 August 1978, 661 mammals of 45 species were collected (Table 53). During 81 nights of trapping 253 marsupials, rodents and carnivores were captured (Table 54). The mist nets collected 251 bats during 20 nights of netting (Table 55). Monkeys, sloths, armadillas and other mammals were collected by hunting. The overgrown Orange grove and field (sites 6 and 17) produced more rodents per trap effort than the forest. The spiny rats (*Proechimys*) were the most common rodents in all sites except the orange grove and field where *Zygodontomys* was the most common.

Serological tests on specimens from these animals are still in progress, and results currently available are incomplete. Antibody to MAY virus was found in sera from eight birds, and to Oropouche (ORO) virus in sera from 64 of 724 birds (Table 56). No viruses were isolated from 737 bird blood specimens tested. Tests on sera from 332 mammals found no antibody to Oropouche virus, and only the primates had antibody to MAY virus.

Among primates, two species were collected, a marmoset, *Callithrix argentata*, and a howler monkey, *Alouatta belzebul*. Among 69 marmosets collected, 13 (19%) had HI antibody to MAY virus. In addition, MAY virus was isolated from an adult female marmoset collected near Vila 129 on 19 April 1978. Only two howler monkeys were collected, and of these, only one had HI antibody to MAY virus. These results are summarized in Table 57.

As an interesting observation, of the 69 marmosets tested, only 4 (6%) had HI antibody to YF virus, and only 2 (3%) had HI antibody to ORO virus. Of the two howler monkeys tested, one had HI antibody to YF virus, but neither had antibody to ORO. Table 57 shows antibody prevalence rates for MAY, YF and ORO viruses by collection site in and around Belterra.

COMMENT: These preliminary results indicate that primates may play a role in the amplification of MAY virus in nature. To further clarify this point, experimental infections are needed to demonstrate that primates circulate virus at a sufficient titer to infect feeding, uninfected vectors. This is attempted in a subsequent section of this report.

TABLE 52 Birds collected in mist nets at the Belterra study area, Pará, Brazil, 15 March through 21 July 1978.

Family	Rubber Plantation Sites A,B,C,F	Lowland Forest Site D	Upland Forest Site E	Total
Accipitridae	1	-	-	1
Columbidae	8	3	4	15
Caprimulgidae	2	-	-	2
Trochilidae	1	-	-	1
Momotidae	-	-	1	1
Bucconidae	-	3	-	3
Dendrocolaptidae	8	8	61	77
Furnariidae	3	1	1	5
Formicariidae	136	36	105	277
Conopophadidae	-	-	5	5
Cotingidae	4	4	2	10
Pipridae	110	28	30	168
Tyrannidae	8	9	14	31
Troglodytidae	9	1	14	24
Turdidae	-	3	3	6
Virionidae	2	-	5	7
Icteridae	-	1	-	1
Parulidae	1	-	-	1
Thraupidae	-	1	3	4
Fringillidae	62	7	13	82
Not Identified	2	-	-	2
TOTAL	357	105	261	723
Mornings netted	35	1	13	53

TABLE 53. Total mammals collected in the Belterra study area, Par , Brazil, 15 March through 25 August 1978.

Species	Total collected	Sera tested
Marsupialia		
<u>Caluromys philander</u>	3	3
<u>Monodelphis brevicaudata</u>	2	2
<u>Marmosa parvidens</u>	1	1
<u>Metachirus nudicaudatus</u>	4	3
<u>Didelphis marsupialis</u>	21	20
Chiroptera		
<u>Micronycteris bracyotis</u>	1	0
<u>Tonatia carrikeri</u>	2	0
<u>T. silvicola</u>	1	0
<u>Phyllostomus latifolius</u>	4	4
<u>Glossophaga soricina</u>	58	5
<u>Linchonycteris abscura</u>	1	1
<u>Carollia brevicauda</u>	81	48
<u>Rhinophylla fischeriae</u>	9	2
<u>R. pumilio</u>	1	0
<u>Sturnira lilium</u>	4	3
<u>Uroderma bilobatum</u>	13	4
<u>U. magnirostrum</u>	1	0
<u>Vampyrops helleri</u>	2	0
<u>Vampyressa bidens</u>	1	0
<u>V. pusilla</u>	2	0
<u>Artibeus cinereus</u>	4	1
<u>A. concolor</u>	5	3
<u>A. fuliginosis</u>	19	16
<u>A. jamaicensis</u>	34	29
<u>A. literratus</u>	48	39
<u>A. sp.</u>	2	0
<u>Desmodus rotundus</u>	1	1
<u>Molossus sp.</u>	2	1
Primates		
<u>Alouatta belzebul</u>	2	1
<u>Callithrix argentata</u>	89	69
Edentata		
<u>Bradypus variegatus</u>	12	11
<u>Cabassus unicinctus</u>	1	0
<u>Dasypus novemcinctus</u>	2	1
Rodentia		
<u>Sciurus gilvigularis</u>	2	2
<u>Oryzomys bicolor</u>	4	1
<u>O. concolor</u>	5	0
<u>O. macconnelli</u>	1	0
<u>Zygodontomys lasiurus</u>	128	28
<u>Oxymycteris sp.</u>	25	2

TABLE 53. Total mammals collected in the Belterra study area, Pará, Brazil, 15 March through 25 August 1978. - Cont.

Species	Total collected	Sera Tested
Rodentia		
<u>Proechimys longicaudata</u>	41	24
<u>P. guyannensis</u>	10	5
<u>Mesomys hispidus</u>	7	2
Carnivora		
<u>Nasua nasua</u>	2	1
<u>Mustela africana</u>	1	1
<u>Eira barbara</u>	2	0
45 species	661	332

TABLE 54. Mammals collected by trapping in the Belterra study area, Pará, Brazil, 15 March through 25 August 1978.

Species	Numbers collected per trapping site								
	1	2	3	4	5	6	7	8	Total
Marsupialia									
<i>Caluromys philander</i>	-	1	-	-	-	2	-	-	3
<i>Monodelphis brevicaudata</i>	-	-	-	1	1	-	-	-	3
<i>Marmosa parvidens</i>	-	-	-	-	1	-	-	-	1
<i>Metachirus nudicaudatus</i>	-	1	-	1	2	-	-	-	4
<i>Didelphis marsupialis</i>	-	-	1	5	9	2	3	-	20
Rodentia									
<i>Oryzomys bicolor</i>	-	-	-	-	3	-	-	1	4
<i>O. concolor</i>	-	-	-	-	2	-	-	3	5
<i>O. macconnelli</i>	-	-	-	-	-	-	-	1	1
<i>Zygodontomys lasiurus</i>	-	-	-	-	-	32	95	1	128
<i>Oxymycterus</i> sp.	1	-	-	-	-	1	23	-	25
<i>Proechimys longicaudatus</i>	1	2	1	11	14	-	2	10	41
<i>P. guyannensis</i>	-	-	4	1	-	-	-	5	10
<i>Mesomys hispidus</i>	-	-	-	-	7	-	-	-	7
Carnivora									
<i>Nasua nasua</i>	-	-	-	-	-	-	-	1	1
<i>Mustela africana</i>	-	-	-	-	7	-	-	-	7
TOTAL	2	4	7	19	39	37	123	22	253
Nights trapped	5	4	7	7	21	9	28*	28*	81*
Trap night	335	232	329	1127	1964	774	582	2176	7.519
Mammals/1000 trap nights	1.0	17.2	21.3	16.8	19.8	47.8	211.3	10.1	33.6

* Trapping areas 7 and 8 were trapped during the same nights.

TABLE 55. Bats collected by mist netting in the Belterra study area, Pará, Brazil, 15 March through 25 August 1978.

Species	Number collected per mist net site							
	1	2	3	4	5	6	G	Total
<u>Micronycteris bracyotis</u>	-	-	-	-	1	-	-	1
<u>Tonatia carrikeri</u>	-	-	1	-	1	-	-	2
<u>T. silvicola</u>	-	-	-	-	1	-	-	1
<u>Phyllostomus latifolius</u>	-	1	2	-	1	-	-	4
<u>Glossophaga soricina</u>	15	23	9	3	-	-	2	52
<u>Lichonycteris abscura</u>	-	-	-	-	1	-	-	1
<u>Carollia brevicauda</u>	6	13	3	14	11	6	3	56
<u>Rhinophylla fischerae</u>	-	1	-	2	-	1	-	4
<u>R. pumilio</u>	-	-	-	-	-	-	1	1
<u>Sturnira lilium</u>	1	-	1	1	-	-	-	3
<u>Uroderma bilobatum</u>	3	1	6	-	1	-	-	11
<u>U. magnirostrum</u>	1	-	-	-	-	-	-	1
<u>Vampyrops helleri</u>	1	-	-	-	1	-	-	2
<u>Vampyressa bidens</u>	-	-	-	-	1	-	-	1
<u>Artibeus cinereus</u>	1	-	-	1	-	-	-	2
<u>A. concolor</u>	-	-	-	-	5	-	-	5
<u>A. fuliginosus</u>	-	-	-	3	14	-	-	17
<u>A. jamaicensis</u>	5	8	2	-	19	-	-	34
<u>A. literatus</u>	-	2	-	-	46	-	-	48
<u>A. sp.</u>	-	-	-	-	2	-	-	2
<u>Desmodus rotundus</u>	-	-	-	-	1	-	-	1
<u>Motossus sp.</u>	-	-	-	-	2	-	-	2
TOTAL	33	49	24	24	108	7	6	251
Nights netted	2	3	3	2	8	1	1	20
Net hours	12	18	21	5	65.5	9	15	145.5
Bats/net hour	2.75	2.72	1.14	4.80	1.65	0.78	0.40	1.72

TABLE 56. Distribution of hemagglutination inhibiting antibody to Mayaro and Oropouche viruses in birds collected in the Belterra study area, Pará, Brazil, 15 March through 21 July 1978.

Family	Mayaro Pos/tested	Oropouche Pos/collected
Accipitridae	0/1	0/1
Columbidae	0/15	0/15
Caprimulgidae	0/2	0/2
Trochilidae	0/1	0/1
Momotidae	0/1	0/1
Bucconidae	0/3	0/3
Dendrocolaptidae	0/75	5/75
Furnariidae	0/5	1/5
Formicariidae	5/265	52/265
Conopophadidae	0/5	0/5
Cotingidae	0/10	0/10
Pipridae	1/163	5/163
Tyrannidae	1/29	1/29
Troglodytidae	0/23	0/23
Turdidae	0/6	0/6
Vironidae	0/7	0/7
Parulidae	0/1	0/1
Thraupidae	0/4	0/4
Fringillidae	1/77	0/77
Chickens	0/28	0/28
Ducks	0/3	0/3
TOTAL	8/724	64/724

TABLE 57. Summary of antibody prevalence rates to Mayaro, Yellow Fever and Oropouche viruses among primates collected in Belterra, Pará, Brazil, April-July, 1978.

Area	Mayaro	Yellow Fever	Oropouche
<u>Callithrix argentata</u>			
Road 5	1/2 (50%)	0/2	2/2 (100%)
Road 6	1/5 (20%)	0/5	0/5
Vila Piquiã	1/12 (8%)	1/12 (8%)	0/12
Vila Viveiro	1/4 (25%)	1/4 (25%)	0/4
Vila 129	9*/39 (23%)	2/39 (5%)	0/39
Sítio Chagas	0/4	0/4	0/4
Southwest Forests	0/3	0/3	0/3
TOTAL	13/69 (19%)	4/69 (6%)	2/69 (3%)
<u>Alouatta belzebul</u>			
Vila Piquiã	0/1	0/1	0/1
Vila 129	1/1 (100%)	1/1 (100%)	0/1
TOTAL	1/2 (50%)	1/2 (50%)	0/2

* Mayaro virus isolated from one marmoset collected from Vila 129 on 19 April 1978.

TABLE 58. Summary of experimental infections of marmosets collected from Belterra, Pará, Brazil and inoculated with 10^3 - 10^4 TCID₅₀/0.1 ml Mayaro virus.

Marmoset	Max. viremia on days post-inoculation					
	2	3	4	5	6	7
<u>Callithrix argentata</u> - 1	10^{4*}	10^2	0	0	0	0
<u>Callithrix argentata</u> - 2	$10^{2.5}$	10^1	0	0	0	0
<u>Callithrix argentata</u> - 3	NT+	0	0	0	0	0
<u>Callithrix argentata</u> - 4	NT	0	dead			
<u>C. humeralifer</u>	10^4	10^2	0	dead		

* TCID₅₀/0.1 ml in Vero cells

+ NT = not tested

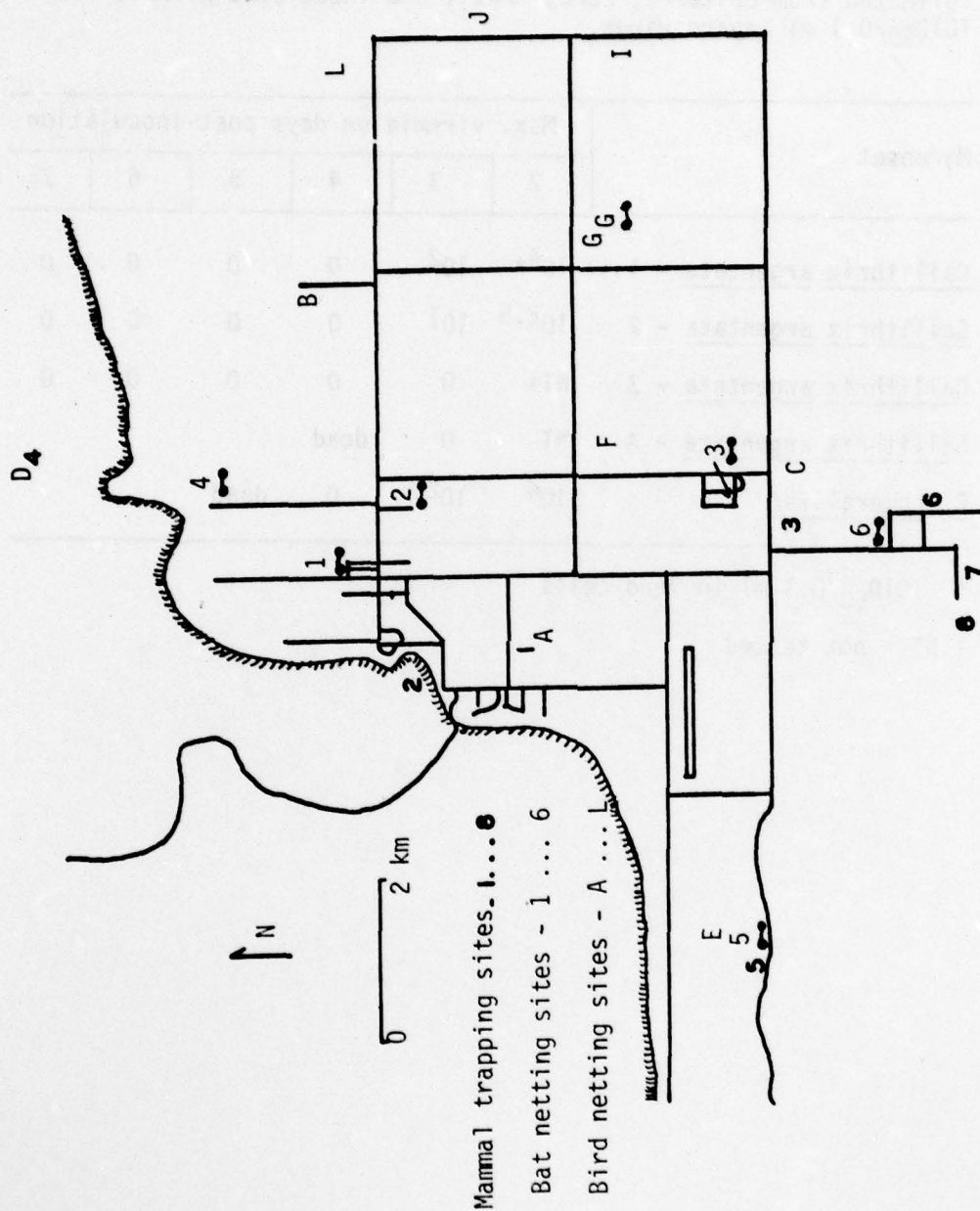


FIG. 24.. Map of Belterra, Pará, Brazil showing the bird and mammal collecting sites.

b. Experimental infections of marmosets

OBJECTIVES: The objective of this section is to determine if marmosets found in Belterra, Pará, Brazil, will produce a significant viremia when infected with Mayaro (MAY) virus.

BACKGROUND: Marmosets, especially Callithrix argentata, are the most common non-human primate found in Belterra. Serological studies on feral marmosets collected in Belterra during the MAY virus outbreak have revealed a high (19%) antibody prevalence rate to MAY virus. In addition, MAY virus was isolated from a viremic, feral C. argentata collected during the outbreak.

All MAY virus isolations made from vectors have been from a single species, Haemagogus janthinomys. Mosquitoes of the genus Haemagogus are known to feed readily on primates, thus an amplification cycle involving Haemagogus mosquitoes and marmosets seems reasonable. For such a cycle to exist, infected marmosets must produce a viremia of sufficient titer to infect feeding uninfected Haemagogus. The objective of this study is to determine the titer of virus circulated by infected marmosets, and thus determine the likelihood of their involvement in an amplification cycle.

DESCRIPTION: Marmosets were collected or purchased in Belterra and transported to Belém alive. Once in the laboratory they were bled to detect pre-existing HI antibodies to MAY virus. Those which lacked antibody to MAY virus were inoculated subcutaneously with 0.2 ml of MAY virus which titered between 10^3 and 10^4 TCID₅₀/0.1 ml in Vero cells grown in tubes. Each marmoset was then bled daily beginning on day 2 or day 3 post-inoculation (p.i.) through day 7 p.i.

Whole blood was drawn and immediately diluted 1:10 in Vero cell growth medium (Medium 199 with 5% fetal bovine serum and antibiotics) and frozen at -70°C pending assay.

Viremia was detected by further diluting each blood sample to 10^{-4} , then inoculating 0.1 ml of diluted blood into duplicate tubes of drained Vero cells. Tubes were incubated for 1 hr at 37°C, then rinsed with 1.0 ml of maintenance medium (medium 199 with 1% fetal bovine serum and antibiotics), and 1.0 ml of maintenance medium added to each tube. Tubes were observed daily for at least 7 days for evidence of cytopathic effect caused by virus. Neutralization tests of virus isolated from viremic marmosets are in progress.

Of the two species of primates tested from Belterra, the marmosets are by far the more common. It would be reasonable to assume that they stand the greater chance of contributing to an amplification cycle, simply due to their numerical abundance. Certainly the isolation of MAY virus from a feral marmoset adds much support to such a hypothesis.

The low antibody prevalence rate to ORO virus among primates is especially noteworthy, since Belterra experienced an outbreak of ORO virus during 1975. Previous serological surveys have detected antibody to ORO virus among primates, and rarely among other animal species. As a result, an endemic cycle which involves primates as an amplifying host has been proposed. However, the results presented here indicate that primates, at least within the areas sampled in Belterra, do not have a high antibody prevalence rate to ORO virus. Either marmosets are relatively short lived, and most exposed during the epidemic of 1975 have already died, or marmosets were not significantly involved in the amplification of ORO virus in the 1975 outbreak. The latter would then support the theory that man is the principal amplifying host in the epidemic cycle of ORO virus as suggested earlier. These results would also suggest that ORO virus is not currently endemic in Belterra.

PROGRESS: A total of 5 marmosets were experimentally infected, 4 *C. argentata* and 1 *C. humeralifer*. Of these, 3 produced a detectable viremia. Of the two marmosets which did not produce a detectable viremia, neither were bled on day 2, but rather daily bleeding began on day 3. One of these died on day 3 as a result of the trauma of bleeding.

Two of the three marmosets viremic on day 2 had titers equal to or in excess of 10^4 TCID₅₀/0.1 ml in Vero cells. The remaining marmoset produced a viremia which titered about $10^{2.5}$ TCID₅₀/0.1 ml in Vero cells. Titers dropped on day 3 to 10^2 TCID₅₀/0.1 ml in 2 marmosets, and 10^1 TCID₅₀/0.1 ml in the other. Viremia was not detected on days 4 through 7 p.i. Table 58 presents a summary of the viremia responses of these 5 marmosets experimentally infected.

COMMENT: It appears from these experimental infections that marmosets are capable of producing a high titered viremia following infection with MAY virus. It seems likely that the two marmosets which failed to produce a viremia may have been viremic on day 1 or 2, but went undetected since they were not bled.

The rapid onset of viremia may have been the results of the high titered inoculum administered. The intensity and duration of viremia following infection through the bite of an infected vector are questions which remain to be answered. Likewise, the titer of viremia needed to infect a feeding vector must be established. Nevertheless, the preliminary results presented here strongly suggest that marmosets may play an active role in the amplification of MAY virus in nature.

C. Studies on the Endemic Cycle

1. Vertebrate host serology from

Cachoeira Porteira

OBJECTIVE: The objective of this section is to identify those feral vertebrate hosts which have been exposed to Mayaro (MAY) virus, as an initial step in identifying the endemic maintenance cycle of this virus.

BACKGROUND: The environs and program of study at Cachoeira Porteira have been presented in detail elsewhere. Briefly, investigations in this area attempt to study the ecology of arboviruses in a tropical forest essentially devoid of human inhabitants.

DESCRIPTION: Collection techniques and processing of serological specimens were described in detail earlier in vertebrate host serology for Oropouche virus. Data included here are from the same sera as described earlier, but for convenience, only those results pertaining to MAY virus have been extracted and presented.

PROGRESS: A total of 508 avian sera were tested for HI antibody to MAY virus. Of these, only two individuals were found positive. Both were members of the family *Bucconidae*, the puffbirds, and both were captured on the Nov-Dec, 1977 trip. Of the 3 puffbirds captured on that trip, 2 were positive for MAY antibody. All 3 puffbirds captured were *Monasa morphoeus*, the white-fronted nunbird. The two positive birds were a male and a female, both captured together. Both had HI antibody titers of 1:40 to MAY virus, and neither had HI antibody to any other Group A arbovirus, so it seems apparent that these results do not represent cross reactions. Neither positive sera has yet been confirmed by neutralization tests. Table 59 presents a summary of birds collected from Cachoeira Porteira and tested by HI for antibody to MAY virus.

Similar to the results presented for Oropouche virus, the only mammals which demonstrated a high prevalence rate of antibody to MAY virus were the primates. Of 37 primate sera tested, 22 (59%) had HI antibody to MAY virus. Of the six species of primates collected, 5 had at least one individual positive. The single species which lacked at least one positive individual, *Pithecia pithecia*, had only one serum tested. All five sera tested of *Alouatta seniculus*, and all three *Ateles paniscus* sera tested were positive. Of 15 *Cebus apella* sera tested, 9 (60%) were positive, and 2 of 3 (67%) *Ateles belzebub* were positive, as were 3 of 10

(30%) Chiropotes satanas sera. Positive sera were found among specimens collected on each of the 3 trips made during 1977. All positive and negative sera have been confirmed by neutralization tests.

Of the other groups of mammals tested, only one Dasyprocta aguti rodent sera of a total of 7 (14%) tested, was positive for HI antibody to MAY virus. Of the remaining 126 rodent sera of other species tested, all lacked HI antibody to MAY virus, as did all 74 marsupial sera, 6 carnivore sera, 15 ungulate sera and 8 other sera. A summary of mammalian sera from Cachoeira Porteira tested by HI for antibody to MAY virus is presented in Table 60.

COMMENTS: White-fronted nunbirds are found from southeastern Honduras to northern Bolivia and Southeastern Brazil. They are most often seen in small groups, and are usually noisy. The nest in Costa Rica is reported to be in a burrow in level ground. Puffbirds as a group are exclusively neotropical and found chiefly in forest and woodlands. Nunbirds in Panama are uncommon to locally fairly common in humid forest and forest borders in lowlands and foothills¹. The finding of 2 of 3 nunbirds collected in Nov-Dec positive for HI antibody to MAY virus deserves further investigation.

An association of MAY virus with birds has been reported previously by Calisher et al.², who isolated MAY virus from an Orchard Oriole, Icterus spurius, migrating into Louisiana in 1967. Serological surveys conducted in the past, however, have very rarely found HI or neutralizing antibody to MAY virus among birds, and the contribution of birds to the maintenance of MAY virus has yet to be resolved.

The results reported here from primates indicate that they are frequently exposed to feeding by the endemic vector of MAY virus. It is possible that the epidemic vector seen in Belterra, Haemagogus janthinomys, is also the principal endemic vector as well, and that primates serve as the main vertebrate host for virus amplification. For such a cycle to be continued indefinitely, some mechanism must be available to the virus for long term maintenance, since primates are relatively few in number and have a low reproductive potential. Potential mechanisms for such maintenance include transovarial transmission by the vector, extreme longevity of the vector, and continual movement of the virus from area to area and population to population. Additional studies are required to determine which of these alternatives, if any, most closely represents the endemic maintenance of MAY virus.

LITERATURE CITED

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TABLE 59. Distribucion of hemagglutination inhibiting antibody to Mayaro virus among birds captured at Cachoeira Porteira, km 71, municipality of Oriximiná, Pará, Brazil, 1977.

Family	Collection period			Total
	March-April	July-August	Nov-Dec	
Alcedridae	-	0/2*	-	0/2
Bucconidae	-	0/1	2/3	2/4
Coeribidae	-	-	0/1	0/1
Columbidae	-	0/1	0/4	0/5
Conopophagidae	0/2	0/3	-	0/5
Cotingidae	-	0/1	0/2	0/3
Cracidae	0/1	-	-	0/1
Dendrocolaptidae	0/16	0/21	0/16	0/53
Falconidae	-	0/1	0/1	0/2
Formicariidae	0/78	0/71	0/45	0/194
Fringillidae	0/1	0/6	0/2	0/9
Furnariidae	0/9	0/17	0/12	0/38
Galbulidae	0/1	0/3	-	0/4
Momotidae	0/7	0/3	0/2	0/12
Parulidae	0/3	-	0/2	0/5
Picidae	0/2	0/1	-	0/3
Pipridae	0/35	0/20	0/5	0/60
Ramphastidae	0/1	-	-	0/1
Sylviidae	0/3	-	-	0/3
Thraupidae	0/6	-	-	0/6
Tyrannidae	0/12	0/9	0/13	0/34
Trochilidae	0/1	-	-	0/1
Troglotididae	0/3	0/5	0/6	0/14
Turdidae	0/13	0/20	0/7	0/40
Vireonidae	-	0/1	-	0/1
	0/201	0/186	2/121	2/501

* Number pos/number tested

TABLE 60. Distribution of hemagglutination inhibiting antibody to Mayaro virus among mammals captured at Cachoeira Porteira, km 71, municipality of Oriximiná, Pará, Brazil, 1977.

Species	March- April	July- August	Nov- Dec	Total
Marsupials				
<u>Didelphis marsupialis</u>	0/2*	0/6	0/6	0/14
<u>Marmosa cinerea</u>	-	-	0/1	0/1
<u>M. murina</u>	0/4	0/3	0/2	0/9
<u>Monodelphis brevicaudata</u>	0/4	0/3	0/10	0/17
<u>Philander opossum</u>	0/9	0/11	0/13	0/33
<u>Total</u>	<u>0/19</u>	<u>0/23</u>	<u>0/32</u>	<u>0/74</u>
Rodents				
<u>Agouti paca</u>	-	0/1	-	0/1
<u>Dasyprocta aguti</u>	-	1/5	0/2	1/7
<u>Hydrochaeris hydrochaeris</u>	-	-	0/1	0/1
<u>Myoprocta acouchy</u>	0/2	-	0/1	0/3
<u>Neacomys spinosus</u>	-	0/3	0/1	0/4
<u>Nectomys squamipes</u>	0/1	0/7	0/1	0/9
<u>Oryzomys bicolor</u>	-	0/1	-	0/1
<u>O. capito</u>	-	0/15	0/7	0/22
<u>Proechimys guyannensis</u>	0/34	0/28	0/21	0/83
<u>Sciurus gilviglires</u>	0/1	0/1	-	0/2
<u>Total</u>	<u>0/38</u>	<u>1/61</u>	<u>0/34</u>	<u>1/133</u>
Primates				
<u>Alouatta seniculus</u>	2/2	-	3/3	5/5
<u>Ateles belzebuth</u>	-	2/3	-	2/3
<u>A. paniscus</u>	1/1	-	2/2	3/3
<u>Cebus apella</u>	-	6/11	3/4	9/15
<u>Chiropotes satanas</u>	-	1/6	2/4	3/10
<u>Pithecia pithecia</u>	-	-	0/1	0/1
<u>Total</u>	<u>3/3</u>	<u>9/20</u>	<u>10/14</u>	<u>22/37</u>
Carnivores				
<u>Eira barbara</u>	0/1	-	-	0/1
<u>Felis concolor</u>	-	0/1	-	0/1
<u>F. pardalis</u>	0/1	-	-	0/1
<u>Nasua nasua</u>	-	0/1	0/2	0/3
<u>Total</u>	<u>0/2</u>	<u>0/2</u>	<u>0/2</u>	<u>0/6</u>

TABLE 60. Distribution of hemagglutination inhibiting antibody to Mayaro virus among mammals captured at Cachoeira Porteira, km 71, municipality of Oriximiná, Pará, Brazil, 1977. - Cont.

Species	March- April	July- August	Nov- Dec	Total
Ungulates				
<u>Mazama americana</u>	0/1*	0/1	0/3	0/5
<u>Tapirus terrestris</u>	-	-	0/1	0/1
<u>Tayassu pecari</u>	-	0/5	0/3	0/8
<u>Dicotyles tacaçu</u>	-	0/1	-	0/1
<u>Total</u>	<u>0/1</u>	<u>0/7</u>	<u>0/7</u>	<u>0/15</u>
Other mammals				
<u>Dasypus novemcinctus</u>	0/1	-	-	0/1
<u>Bats</u>	-	0/5	0/1	0/6
<u>Tamandua tetradactyla</u>	-	-	0/1	0/1
<u>Total</u>	<u>0/1</u>	<u>0/5</u>	<u>0/2</u>	<u>0/8</u>

* Number pos/number tested

III. MULTIDISCIPLINARY DISEASE SURVEILLANCE ALONG THE TRANSAMAZON HIGHWAY, PARA, BRAZIL.

A. Entomological Surveillance

OBJECTIVE: The current program attempts to isolate virus from mosquitoes collected between 1975 and 1976 from sites along the Transamazon, Highway, Pará, Brazil.

BACKGROUND: A program of entomological surveillance was conducted at 12 sites along the Transamazon Highway from 1974 to 1976. Main emphasis in the schedule of collections centered around sampling those insects attracted to man. In addition, insects were also collected using Shannon traps and Chamberlain light traps. Details of the more general objectives, collecting methods and daily schedules have been presented in the Annual Reports of FY 1975 and 1976. Descriptions of the 12 collecting sites can be found in the Annual Report of FY 1977. That report also contains a detailed discussion of the temporal and spatial diversity and/or similarity of the mosquito fauna along the Transamazon Highway.

This report represents the final analysis of entomological data collected along the Transamazon Highway, the assay of insects for the presence of virus.

DESCRIPTION: Insects were identified and pooled in groups of approximately 50 or fewer individuals. Identification was made to species whenever possible; if insects lacked sufficient taxonomic characteristics for specific identification, they were pooled by subgenus or genus. Engorged individuals were not included in groups assayed for virus. Groups of insects were triturated in chilled tissue grinders with 1.5 ml of diluent which contained: 20% bovine albumin, penicillin, streptomycin, gentomycin and fungizone in phosphate buffered saline. Triturated pools were centrifuged for 15 minutes at 2000 RPM, then 0.1 ml of supernate inoculated into duplicate tubes of Vero cell culture. Tubes were observed daily for 7 days. Suspect positive tubes were frozen (-70°C) and subsequently passed to other tubes of Vero Cell culture.

PROGRESS: A total of 9920 mosquitoes in 256 pools have been assayed for virus in Vero cell. These mosquitoes represented collections made at Gleba 05, lote 05; Gleba 29, lote 03; Gleba 38, lote 6; Gleba 57, lote 03; Gleba 66, lote 09; and Palestina. A summary of the number of mosquitoes and pools tested from each area is presented in Table 61. Mosquitoes tested represented 12 different genera and 30 species. An additional 10 subgeneric or generic categories were needed for those individuals which could not be identified to species. A synopsis of the number of mosquitoes tested by species and locality is presented in Table 62. No virus was isolated from any mosquito pool tested.

COMMENT: A number of explanations are possible for our failure to isolate virus from these mosquitoes tested. Certainly too few have been tested to conclude beyond a doubt that no viruses were active at the time these samples were made. Also, some viruses known to occur in the area do not cause cytopathic effect (CPE) in Vero cells. These are, however, identified for the most part, and are mostly viruses which are generally not of public health importance.

Recent experience in this laboratory indicates that the storage facilities currently in use in our laboratory are not suitable for preserving virus infectivity for extended periods. This is based on the following observations. A low passage stock of Mayaro (Togaviridae, Alphavirus) virus was prepared on 16 June 1978 and stored in medium 199 with 20% fetal bovine serum as 0.5 ml aliquots. This stock was titrated on 21 June 1978 and it was determined that a dilution of 1:4 of 10^{-5} would result in approximately 100 plaque forming units (pfu) in Vero cells grown in 25 cm² flasks. An experiment done on 28 June 1978 resulted in a dose of 32 pfu when this stock was diluted 1:4 of 10^{-5} . Further drops in titer were recorded in each subsequent experiment. Tests done on 4 August resulted in a low dose and would have required a dilution of 1:4 of 10^{-4} to produce 100 pfu, equivalent to a drop in titer of 1 log. A test on 25 Aug, again had a low dose, and would have required a dilution of 1:2 of 10^{-3} to produce 100 pfu. This is a drop in titer in excess of 2 logs in little over 2 months storage.

All virus stocks were stored in an upright Revco freezer set at -70°C. Remaining virus in each aliquot opened was discarded after use, so no aliquot of virus was used more than once. However, the power supply for Belém is extremely erratic, and power is frequently cut for extended periods. Consequently, it seems likely that this stock virus has unknowingly been warmed several times. Since all insect pools collected from the Transamazon Highway project have been stored in this same freezer, it seems likely that any virus once present in these insects is no longer viable. Therefore, processing of these pools is being discontinued.

TABLE 61. Total number of mosquitoes and pools assayed for virus in Vero cell culture by locality of collection along the Trans - amazon Highway, Pará, Brazil. Mosquitoes were collected in 1975-76 and tested in 1978. No virus was isolated from any pool tested.

Location	Total tested	Total pools
Gleba 05, lote 05	1399	37
Gleba 29, lote 03	1683	42
Gleba 38, lote 06	337	15
Gleba 57, lote 03	145	8
Gleba 66, lote 09	1650	54
Palestina	4706	100
Total	9920	256

TABLE 62 Summary of mosquito species assayed for virus in Vero Cell culture by location of collection. Mosquitoes were collected during 1975-76 and assayed in 1978. No virus was isolated from any pool tested.

Species	Gleba 05 Lote 05	Gleba 29 Lote 03	Gleba 38 Lote 06	Gleba 57 Lote 03	Gleba 66 Lote 98	Palestina
<i>Aedes serratus</i>	-	56 (1)*	-	-	-	-
<i>Ae. scapularis</i>	-	49 (1)	-	-	-	-
<i>Anopheles albitarsis</i>	-	-	-	-	-	156 (3)
<i>An. aquasalis</i>	-	-	-	-	-	39 (1)
<i>An. mediopunctatus</i>	-	-	-	-	7 (1)	-
<i>An. nuneztovari</i>	282 (6)	29 (1)	-	-	230 (5)	-
<i>An. oswaldoi</i>	38 (1)	36 (1)	10 (1)	-	267 (6)	-
<i>An. triannulatus</i>	377 (8)	200(6)	-	-	337 (8)	655(14)
<i>An. sp.</i>	-	-	-	-	103 (3)	311 (6)
<i>Coquillettidia nigricans</i>	32 (1)	-	-	-	-	40 (1)
<i>Coq. venezuelensis</i>	35 (1)	78(2)	-	-	61 (2)	715(15)
<i>Culex corniger</i>	-	-	-	-	-	236 (5)
<i>Cx. coronator</i>	32 (1)	362(8)	123 (3)	12 (1)	67 (3)	187 (4)
<i>Cx. fatigans</i>	-	-	-	-	-	1004(20)
<i>Cx. taeniopus</i>	-	-	-	-	8 (1)	-
<i>Cx. (c) sp.</i>	93 (3)	63(2)	13 (1)	7 (1)	49 (1)	449(9)
<i>Cx. (m) sp.</i>	-	-	-	-	37 (2)	84(2)
<i>Cx. sp.</i>	-	73(2)	76 (2)	23(1)	214 (5)	52(1)
<i>Haemagogus leucocelaenus</i>	-	-	-	-	14 (2)	-
<i>Hg. sp.</i>	-	12(1)	9 (1)	35(1)	55 (2)	-
<i>Limatus durhamii</i>	10 (1)	-	-	-	-	-
<i>Mansonia humeralis</i>	34 (1)	-	-	-	-	-
<i>Ma. titillans</i>	163 (4)	11(1)	-	-	8 (1)	227(6)
<i>Ma. spp.</i>	-	-	-	-	-	56(2)
<i>Psorophora cingulata</i>	20 (1)	202(4)	-	-	10 (1)	147(3)
<i>Ps. ferox</i>	-	99(3)	-	-	-	-
<i>Sabethes belisarioi</i>	35 (1)	10(1)	-	-	3 (1)	-
<i>Sa. chloropterus</i>	77 (2)	40(2)	23 (2)	17(1)	64 (2)	-
<i>Sa. cyaneus</i>	-	-	-	-	3 (1)	-
<i>Sa. glaucodaemon</i>	13 (1)	19(1)	11 (1)	-	56 (2)	-
<i>Sa. tarsopus</i>	-	-	-	-	10 (1)	-
<i>Sa. sp.</i>	46 (1)	-	-	6(1)	-	-
<i>Trichoprosopon digitatum</i>	-	-	23 (1)	45(2)	-	-
<i>Tr. sp.</i>	-	-	15 (1)	-	-	-
<i>Uranotaenia calosomata</i>	29 (1)	41(1)	-	-	6 (1)	-
<i>Ur. geometrica</i>	-	150(3)	-	-	14 (1)	-
<i>Ur. lowii</i>	-	-	-	-	-	13(1)
<i>Ur. sp.</i>	22 (1)	53(1)	18 (1)	-	20 (1)	325(7)
<i>Wyeomyia aporonomia</i>	25 (1)	-	-	-	-	-
<i>Wy. sp.</i>	36 (1)	-	16 (1)	-	7 (1)	-
TOTALS	1399 (37)	1683(42)	337 (15)	145(8)	1650(54)	4706(100)

* Number tested (number of pools)

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION NO.		DATE OF SUMMARY		REPORT CONTROL SYMBOL	
				DA OC 6447		78 10 01		DD-DR&E(AR)355	
2. DATE PREV SUMMARY		3. KIND OF SUMMARY		4. SUMMARY SCTY		5. WORK SECURITY		6. READING	
77 10 01		D. Change		U		U		NA NL	
7. NO. / CODES		8. PROGRAM ELEMENT		9. PROJECT NUMBER		10. TASK AREA NUMBER		11. WORK UNIT NUMBER	
		62770A		3M162770A802		00		011	
12. CONTRIBUTING		13. CONTRIBUTING		14. CONTRIBUTING		15. CONTRIBUTING		16. CONTRIBUTING	
		CARDS 114F							
17. TITLE (Precede with Security Classification Code)									
(U) Health Care and Management of Laboratory Animals									
18. SCIENTIFIC AND TECHNOLOGICAL AREA									
010100 Microbiology									
19. START DATE		20. ESTIMATED COMPLETION DATE		21. FUNDING AGENCY		22. PERFORMANCE METHOD			
76 07		CONT		DA		C. In-House			
23. CONTRACT/GRANT				24. RESOURCES ESTIMATE		25. PROFESSIONAL MAN YRS		26. FUNDS (in thousands)	
a. DATES/EFFECTIVE: NA				b. PREVIOUS		c. CURRENT		d. FUTURE	
b. NUMBER				78		2.5		386	
c. TYPE				79		3.5		526	
d. KIND OF AWARD				f. CUM. AMT.					
27. RESPONSIBLE DOD ORGANIZATION					28. PERFORMING ORGANIZATION				
NAME: Walter Reed Army Institute of Research					NAME: Walter Reed Army Institute of Research				
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RESPONSIBLE INDIVIDUAL					PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				
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29. GENERAL USE					30. ASSOCIATE INVESTIGATORS				
Foreign Intelligence not considered					NAME: Binn, Leonard N., PhD				
31. REVISIONS (Precede each with Security Classification Code)									
(U) Disease surveillance; (U) Klebsiella pneumoniae; (U) Aotus; (U) Random-source dogs; (U) Viral respiratory disease; (U) ELISA									
32. TECHNICAL OBJECTIVE									
23. (U) To investigate diseases and/or conditions affecting laboratory animals used specifically for military research to enhance production quality and health management and to provide research animals free of known or potential pathogens. The ability to provide clinical diagnosis to laboratory animal problems peculiar to the WRAIR animal colonies is critical to the specific research conducted by the WRAIR. The establishment of a disease data storage/retrieval system will provide unique epidemiological information not available from any other laboratory source.									
24. (U) Conventional epidemiologic, pathologic, and microbiologic methods are employed; unconventional procedures are developed as needed.									
25. (U) 77 10 - 78 09 The data storage/retrieval system is in the first generation of editing and development. Aotus were shown to harbor various biotypes and serotypes of Klebsiella pneumoniae, including serotype 2. Type 2 antigen and antibody were detected in the plasma of some monkeys, and, on occasion, the antigen and IgG were found on the monkeys' RBCs. Antibiotic sensitivity correlated with indol neg animal isolates, while resistance correlated with indol pos animal isolates. Cytomegalo-like viruses were isolated from throat secretions and salivary glands of 50 percent of the Aotus examined, and could be reisolated from infected monkeys for at least 1 year. More than 75 percent of the Aotus had neutralizing antibodies to the reference isolate. Additionally, 3 adenoviruses were isolated from moribund Aotus, with greater than 80 percent of the Aotus exhibiting neutralizing antibodies. The ELISA assay was found to be effective in detecting serum antibodies to canine distemper virus (CDV). The ELISA assay for CDV-specific IgM and IgG correlated with serum neutralization tests. Five of 7 foxhound dogs with diarrheal disease yielded coronaviruses and had increased serum neutralization antibody titers. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.									

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Task 00 Military Animal Resources Development

Work Unit 011 Health Care and Management of Laboratory Animals

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Description:

Investigate diseases and/or conditions affecting laboratory animals used specifically for military research to enhance production quality and health management and to provide research animals free of known or potential pathogens.

During the reporting period, research activities have included:
(1) investigation of microbiological aspects, bacterial and viral, of morbidity and mortality among monkeys of the WRAIR Aotus colony,
(2) development and evaluation of an enzyme-linked immunosorbant assay for humoral antibodies to canine distemper virus, and (3) definition of a diarrheal disease among foxhound dogs.

1. Clinical evaluation of disease problems affecting Aotus monkeys.

During the past year, 27 of 210 juvenile, adolescent, and adult owl monkeys died, yielding an annual mortality rate of 13%. The majority of these animals succumbed to glomerulonephritis, regenerative anemia, septicemia, or a combination thereof. In an effort to better define the pathologic condition(s) or diseases(s) affecting owl monkeys, completed case reports of 37 juvenile, adolescent, and adult owl monkeys which died between 1 January 1977 and 1 May 1978 (a 17 month period) were studied. The review included all available ante- and postmortem data, to include clinical histories, clinical pathology reports, histopathology reports, and bacteriology, virology, and serology reports. The purpose of this report is to summarize the observations and interpretations.

Of the 37 dead monkeys, 17 were males and 20 were females. The exact age at the time of death was known for 5 of the monkeys because they had been born in the WPAIR Aotus colony. Four of these were 10 to 16 months of age and the fifth was 4 years old (Table 1). The age distribution of the remaining 32 monkeys was estimated by the amount of dental wear and reflected the approximate age distribution of the entire colony. Distribution of karyotypes among the dead monkeys was similar to the karyotype distribution of the colony. Thus, it appeared that mortality was unaffected by age, sex, or karyotype of the animal. The mortality rate was essentially the same in each of the colony breeding rooms; however, occasional relocation of monkeys precluded definitive evaluation of this observation.

Clinical histories indicated that 21 monkeys exhibited no clinical signs of illness before onset of the terminal illness. Five monkeys had been treated for minor problems: 3 for conjunctivitis, 1 for a vaginal infection, and 1 for a lacerated knee. Nine monkeys had clinical signs that may have reflected more serious disease, to include weight loss (3), abortion (1), depression (1), facial edema (1), anemia (1), and air sacculitis (2). The remaining 2 monkeys had been splenectomized 17 and 35 days before death. Clinical signs observed during terminal illness were lethargy and depression (17), facial edema (2), ascites (2), pale mucous membranes (5), severe weight loss (3), and swollen air sacs (3). Eight animals died without detectable signs, and 3 monkeys were euthanized for a special renal study in which monkeys suspected of having varying degrees of glomerulonephritis were selected.

The primary condition associated with death of the 37 monkeys was assessed by the attending veterinarian after examination of the clinical and pathological data (Table 2). Only 1 cause of death was listed. Therefore, when more than one disease process occurred simultaneously, the pathologic process which appeared to contribute most to the animal's terminal condition was considered the primary cause of death. Three most common diagnoses were renal disease (15), anemia (9), and septicemia (5). The remaining 8 animals died of other causes: euthanasia (3), gentamycin toxicity (2), chronic meningoencephalitis (1), anoxia (1), and unknown (1).

The histopathologic lesions observed most frequently in the 37 monkeys are shown in Table 3. Varying degrees of glomerulonephritis (GN) were found in 34 of these monkeys. The GN lesions were classified as severe in 12 monkeys, moderate in 13, and mild in 9. Glomerular fibrosis, which was observed in 24

monkeys, demonstrated the chronicity of the glomerular disease. Interstitial nephritis was seen in 31 monkeys and may have been a secondary lesion to the other renal pathology.

Table 4 shows the severity of the GN lesions in animals within various cause-of-death categories. As would be expected, animals that died of renal disease had the highest incidence of severe and moderate GN lesions. Of the 9 monkeys in which anemia was the predominant pathologic change, 7 had detectable GN lesions (2 severe, 3 moderate, and 2 mild), while 2 monkeys had essentially normal glomeruli. Glomerular lesions in animals that died of septicemia were mild (4) or moderate (1).

Anemia was a significant finding among the 37 monkeys. Routine blood analyses performed when the monkeys appeared healthy indicated that 14 of the monkeys had red blood cell (RBC) counts below 4×10^6 per cmm of blood. Blood samples obtained from 22 monkeys during terminal illness showed 15 were anemic. Of the 9 monkeys in which anemia was the predominant sign at death (Tables 2 and 4), 6 had blood samples withdrawn within 24 hours of death. The RBC counts from these 6 samples ranged from 0.56 to 1.74×10^6 RBCs per cmm of blood. Fourteen monkeys in this study did not develop evidence of anemia.

Histologically, 30 of 33 bone marrows examined were hyperactive, and 21 of 37 monkeys had histologic evidence of extramedullary hematopoiesis (Table 3). These findings indicated that there was a definite attempt to compensate for erythrocyte loss. Since anemia in humans with chronic renal disease usually is of a hypoplastic type, it would appear that anemia and GN in *Aotus* monkeys are attributable to indirect mechanisms. Elucidation of the explicit causative mechanisms will have to await further studies. However, pathologic immune response may be involved, as indicated by the high incidence of erythrophagocytosis and reticuloendothelial hyperplasia (Table 3).

Klebsiella pneumoniae was the predominant bacterial pathogen isolated. The organism was isolated from the throat and/or rectum of 13 of 19 monkeys cultured antemortem. Serotyping of these isolates was not performed because of the high incidence of *K pneumoniae* that was detected on food stuffs provided the monkeys. At necropsy, lung, liver, kidney, and spleen were cultured from 29 of the 37 monkeys. Seven animals (24% of those cultured) had *K pneumoniae* isolated from two or more of the tissues. All of the isolates from organ tissues which were typeable were pathogenic serotype 2. *K pneumoniae* was the

only organism repeatedly isolated from 4 cases of purulent air sacculitis.

Septicemia was considered the primary cause of death in 5 of the 37 monkeys (Table 2). Three were confirmed at necropsy to be caused by K pneumoniae infection. Antemortem evidence, isolation from blood, and purulent air sacculitis implicated K pneumoniae in a fourth case. One animal died of broncho-pneumonia, apparently caused by Bordetella bronchiseptica. Additional studies have been implemented to determine the role of K pneumoniae in causing disease among owl monkeys.

Virus isolation was attempted from throat and rectal swabs and necropsy tissues of 28 of the 37 owl monkeys. A cytomegalovirus (CMLV) was isolated from throat swabs and/or histologically normal salivary glands from 12 (43%) of the monkeys surveyed. Four other animals, for which virus isolation attempts were negative, had serum neutralizing antibodies (1:8 to 1:16 titers) against 1 of the CMLV isolates. Aside from 1 positive isolation from a spleen, attempts to isolate CMLV from rectal swabs or from other internal organs were negative. An adenovirus was isolated from rectal swabs from 2 of the monkeys. Definition of the relationship of these viruses to disease conditions observed in owl monkeys requires additional investigation.

2. Epizootiology of Klebsiella in Aotus monkey colonies.

Klebsiella pneumoniae, especially capsule serotypes 1 and 2, is a recognized natural pathogen of nonhuman primates. The bacterium often is incriminated as one of the major microorganisms associated with morbidity and mortality in nonhuman primate colonies. Although its importance and attack rate may vary with the host species and colony, the overall significance of this microbe has been recognized as it is 1 of 6 bacteria that was provided a specific category for reporting in the Center for Disease Control Primate Zoonoses Surveillance Report. In recent years, serotyping has shown that K pneumoniae type 2 was the bacterial serotype isolated most often from necropsy specimens.

Records of the Aotus monkey colonies at the Walter Reed Army Institute of Research (WRAIR) indicated that gram negative bacteria, especially K pneumoniae, often were associated with sickness and/or death in Aotus monkeys. The problem continued to increase, whereupon a special epizootiologic study was devised for the nonbreeding portion of the WRAIR Aotus colony.

The findings were correlated and, whenever applicable, compared to other Aotus populations within the WRAIR.

For study purposes, the WRAIR Aotus colony was readily divisible into 3 subcolonies based on location and type of Aotus housed. Subcolony 1 was composed of 35 non-breeding owl monkeys that were caged individually in Room 202A of Bldg 511. Monkeys of this group were the most intensively studied. Subcolony 2 consisted of 2 rooms (202J and 202K, Bldg 511) of breeding pairs and their offspring. Each breeding pair with offspring were housed together in a single cage. Subcolony 3 contained approximately 40 owl monkeys being used for malaria research and was located in Room 4043 of Bldg 40. This latter subcolony was about 3.5 miles distant from the former 2 subcolonies.

Klebsiella survey of monkeys in Room 202A. Throat and rectal specimens were obtained with sterile swabs from anesthetized monkeys. Fecal samples were collected from pans under the wire floor of each cage. All of the monkeys were surveyed 5 times at 3 major intervals during the 7 month period between September 1977 and March 1978. A minimum of 1 each throat, rectal, and fecal sample was obtained from each monkey at each interval. Monkeys determined to be carriers of K pneumoniae were examined at intermediate times between major intervals. During the first 60 days, 12 of 35 Aotus monkeys were found to harbor K pneumoniae. The number of culture positive monkeys was extended to 16 by the end of the study.

An attempt was made to eradicate K pneumoniae type 2 from 2 chronic carrier monkeys. One monkey consistently yielded K pneumoniae type 2 from both throat and rectal samples, while positive cultures were obtained only from rectal samples of the second monkey. Since the isolates recovered were sensitive to cephalothin, the monkeys were treated with 50 mg sodium cephalothin BID intramuscularly for 7 days. The monkeys were examined culturally during and 11 days after treatment. The rectal carrier monkey yielded repeated isolates of K pneumoniae type 2 during and after treatment. Isolates were not obtained from the throat and rectal carrier monkey on days 7 and 8 after treatment started; however, K pneumoniae type 2 was isolated from the throat and rectum of this animal 11 days after treatment stopped. The results suggested that the antibiotic did not attain therapeutic levels where the organisms were colonized or that the animals were reinfected by external vectors. Also, the suppression of K pneumoniae type 2 in 1 monkey was not sufficient to permit complete clearing or to prevent reinfection.

The prevalence of K pneumoniae in throat specimens varied from 3 to 15%, and ranged from 10 to 33% in rectal specimens on various days during the 7 month study. Almost half (16/35) of the monkeys had K pneumoniae recovered from throat or rectal specimens during this time. Eight monkeys had Klebsiella isolated from both throat and rectal swabs, while the other 8 monkeys yielded only rectal isolates. There were no monkeys which had only throat isolates.

The K pneumoniae isolates were separable into three major groups: indole positive non-capsular type 2, indole negative capsular type 2, and indole negative non-capsular type 2.

Ten indole positive strains were isolated from 7 different monkeys. Of these isolates, 80% were recovered during a 7 day period, (26 October to 2 November 1977). This suggested a temporal introduction of organisms which were transients in the monkeys or a resurgence of indigenous organisms. The indole positive capsular types included serotypes 68, 64, and 54, as well as 4 isolates which were untypeable due to insufficient capsules.

A total of 40 isolates of indole negative capsular type 2 were recovered from 4 monkeys. In all, well over half (50/74) of the total Klebsiella sp isolations were capsule type 2. This suggested that capsular type 2 organisms had the ability to colonize certain Aotus monkeys, resulting in chronic infections.

Indole negative non-capsular type 2 isolates were recovered from 13 of 35 monkeys for a total of 24 isolates. These isolations were sporadic in occurrence. Most of the isolates produced insufficient capsules for typing. One isolate was capsular type 8. Eight monkeys yielded 2 of the 3 different classes of Klebsiella (indole positive, indole negative type 2, indole negative non-type 2).

Antibiotic sensitivities. Isolates from each monkey were tested for antibiotic sensitivity, and sensitivity patterns were correlated with the 2 major biotypes. All 10 indole positive strains were multiply resistant to antibiotics (Table 5). These strains were sensitive to gentamycin, trimethoprim, and furadantoin; intermediate in sensitivity to cephalothin; and resistant to chloramphenicol, streptomycin, ampicillin, carbenicillin, sulfas, and tetracycline. The indole negative isolates, consisting of capsular types 2 and 8 and untypables, were generally resistant to ampicillin and carbenicillin, but were susceptible to gentamycin, cephalothin, sulfas, trimethoprim,

tetracycline, streptomycin, and furadantoin. One exception was isolate 27-10-27, a non-type 2 indole negative strain, which manifested the same antibiogram as the indole positive monkey isolates (Figure 1, Table 5).

Isolation of *K pneumoniae* from the environment. The persistence of capsular type 2 strains after antibiotic treatment and the transient occurrence of indole positive strains suggested *K pneumoniae* was being introduced into the colony. Previous investigations had recovered *K pneumoniae* from such diverse environments as drinking water, vegetables, fruits, and sawdust bedding (1,2). In the present study, *K pneumoniae* was isolated from orange surfaces, commercial monkey chow, an animal fattening food, a human baby food used to make a liquid supplement, cockroaches, and monkey water bowls. Isolates of *K pneumoniae* were not recovered from apple surfaces or the throats of any of the veterinary technicians. Table 6 lists the environmental isolates and their antibiotic sensitivities. It was remarkable that the strains recovered were so homogenous in their antibiotic sensitivities, regardless of indole biotype. In fact, these isolates were sensitive to all of the antibiotics tested, except ampicillin. The antibiotic sensitivities of our indole positive environmental strains were similar to the indole positive clinical strains of Nishida et al (3).

The indole positive isolates from the owl monkeys were very resistant to antibiotics, which contrasts with the reported findings of other indole positive clinical strains (3,4). The possibility exists that other environmental strains are antibiotic resistant, and it is tempting to speculate that a transfer of plasmid resistance factor may be the reason the indole negative clinical isolate 10-27-10 was multiply and highly resistant to antibiotics (Table 5). As a consequence, the ingestion of extraneous strains of *K pneumoniae* could readily confuse bacterial diagnosis of disease. There also is the possibility that in a dual infection the non-pathogenic strains could either destroy antibiotics or transfer antibiotic resistance factors to virulent strains.

Comparison of the three *Aotus* subcolonies. Throat samples were obtained randomly from 52 of the monkeys housed in Room 202J of Bldg 511, and throat and rectal samples were obtained from 15 of the monkeys housed in Bldg 40. The specimens were screened for *Klebsiella* sp in particular and enterobacteria in general. *Klebsiella pneumoniae* was recovered from 10 of the 52 owl monkeys in Room 202J. Many of the monkeys examined also har-

bored organisms of numerous other Enterobacteriaceae in their throats. Of the monkeys examined from Bldg 40, none yielded Klebsiella sp from throat or rectal specimens. Also, bacteria in the Enterobacteriaceae family were not detected in throat samples. The predominant bacteria recovered from rectal specimens of owl monkeys in Bldg 40 were Proteus sp and E coli.

On the basis of previous studies it was concluded that coliforms and Proteus bacterial species were not consistent inhabitants of the Aotus throat. It was our opinion that the ability to colonize the throat may be in part due to the adhesiveness of the organism, individual immunity among monkeys, and environmental influences.

The most marked differences observed among the 3 subcolonies were the prevalence of Klebsiella and other Gram negative organisms in the throats of "normal" Aotus monkeys in Bldg 511 and an increased incidence of illness and death in Bldg 511. During the 7 month study, no deaths, other than those attributed to investigative procedures, occurred in the Bldg 40 subcolony or in Room 202A, whereas approximately 20 deaths occurred in Rooms 202J and 202K.

Aside from the obvious geographic differences, other differences among the subcolonies were sought and noted. The air in Room 202J usually contained a greater concentration of NH_3 (sometimes as high as 14 ppm) than Room 202A or Room 4043³ in Bldg 40 (1 to 2 ppm). Management practices also varied. Water bowls, Shell no-pest strips, and, occasionally, highly concentrated phenol disinfectants were used in Bldg 511, whereas hanging water bottles, no hanging pest strips, and no concentrated disinfectants were the practice in Bldg 40. The number of monkeys per cage generally was lower in Bldg 40.

Detection of subcellular Klebsiella antigens and antibodies in the blood of Aotus monkeys. Plasma and erythrocytes were obtained from 15 monkeys in Room 202A which were known to have had Klebsiella infections (3 that had K pneumoniae type 2 and 12 that had K pneumoniae non-type 2). The plasma was examined by counter current electrophoresis (CCE) for the presence of circulating antibody and antigen. Limulus amoeba lysates (LAL) also were used to detect circulating endotoxin. The antigen used in CCE was purified type 2 capsular material. The antibody used to detect soluble klebsiella antigens was hyper-immune rabbit serum prepared against a whole bacterial cell antigenic preparation. Only 1 monkey (8142) exhibited circulating antigens and antibody to Klebsiella pneumoniae and had

a weakly positive LAL test.

Five of the 15 monkeys had circulating antibody to type 2 capsular material. Two of the seropositive monkeys had had Klebsiella infections caused by serotype 2, while the remaining 3 seropositive monkeys had previously yielded Klebsiella isolates of serotypes other than type 2.

One of the 3 monkeys (8142) that had yielded type 2 organisms developed an antibody response to type 2 capsular material, and attempts to reisolate type 2 organisms were unsuccessful after a 7 month interval. Klebsiella pneumoniae type 2 frequently was isolated from throat and rectal specimens of a second monkey (8162); however, an antibody response was never detected and the organisms continued to persist throughout the study. By contrast, K pneumoniae type 2 organisms were routinely recovered from rectal specimens of monkey A105, even though a seropositive antibody response to type 2 capsular material had developed. This data indicated that the immune response of owl monkeys to K pneumoniae type 2 organisms was inconsistent. Consequently, attempts to eliminate endemic foci of K pneumoniae via immunization need to be investigated under rigorously defined conditions.

Since a significant proportion of the owl monkeys in the WRAIR colony have exhibited signs of anemia, an attempt was made to detect capsular type 2 antigen and Aotus IgG on the erythrocytes of the 3 monkeys chronically infected with K pneumoniae type 2. Rabbit antisera to whole cell K pneumoniae type 2 did not cause agglutination of the erythrocytes. Addition of guinea pig sera (GPS) containing complement to the mixture, however, resulted in lysis of erythrocytes from monkey 8142. The erythrocytes were not lysed with GPS alone, thus ruling out the existence of heterophile antibodies. These observations indicated that antigen coated erythrocytes may be involved in the production of hemolytic anemia in some monkeys. When rabbit antisera to Aotus IgG was reacted with erythrocytes from the 3 monkeys no reaction occurred, even in the presence of GPS. However, the erythrocytes from several other monkeys did react with the anti-IgG and GPS complement, suggesting another possible cause of hemolytic anemia.

Clinical pathology parameters. The clinical pathology profile of owl monkeys was determined and compared among 3 different groups: (1) 3 monkeys chronically infected with K pneumoniae type 2, (2) 13 monkeys that yielded sporadic isolations of various Klebsiella sp, and (3) 18 monkeys that were consis-

tently free of K pneumoniae. Clinical parameters measured included erythrocytes/cmm of blood, mg of creatine/dl of serum, mg of blood urea nitrogen/dl of serum, and gm of albumin/dl of serum. Each of the parameters was measured at the initiation and the termination of the 7 month study. For these groups of owl monkeys, a significant difference was not observed in the average erythrocyte counts or serum chemistry values among the 3 monkey groups (Table 7).

Conclusion. Klebsiella pneumoniae serotype 2 was determined to be the primary bacterial pathogen among owl monkeys in the WRAIR colony. During a 7 month epizootiologic study, many other indole positive and indole negative strains were isolated. The former organisms appeared to be transient within the colony and confused the overall K pneumoniae pathogenesis picture. The pathogenicity potential of such strains remains undefined. Management procedures should be implemented immediately to effectively reduce the populations of transient Klebsiella sp. Management, chemotherapeutic, and immunologic methods will be investigated to define the most appropriate means of eliminating K pneumoniae type 2 infections from the colony.

3. Virus studies of adult and infant Aotus monkeys.

The initial studies to isolate and identify viruses from Aotus monkeys with renal and/or anemic diseases were reported (5). Based on the type of cytopathic effect (CPE) observed, the recovered agents were placed into 3 groups. During this reporting period, additional viral isolations were made from post-mortem tissues of adult and infant owl monkeys. Also, studies were performed to characterize and determine the significance of the viruses. The percentage of owl monkeys with humoral antibodies against these viruses was ascertained. The purposes of this report were to summarize and evaluate the findings of these studies.

One of the 3 types of CPE observed resembled that of the herpesviruses, and was caused by the viruses recovered from throat and rectal swab specimens of a monkey (8147) which died with a generalized herpetic infection (5). Using serum neutralization tests, the isolates were identified as Herpesvirus simplex. The monkeys in the WRAIR Aotus colony had been given attenuated H simplex vaccine. Additional monkeys infected with this virus have not been detected; therefore, H simplex does not appear to be a problem in the WRAIR Aotus colony.

The second group of cytopathic agents produced a characteristic, slowly progressing, focal CPE. These agents were identified as cytomegalo-like viruses (CMLV) after optical and electron microscopic examination of infected cell cultures (5). During the past 12 months similar viruses were recovered frequently from the throat of 86% (37/43) of the apparently normal monkeys examined, and from the salivary glands of 52% (17/33) of the adult monkeys that died (Table 8). The frequency of isolation of CMLV from infant owl monkeys was significantly less. Except for the oropharyngeal cavity, the only sources of CMLV isolation have been from the spleen of 1 monkey that died and from the leukocytes (WBC) of an apparently healthy monkey. In both of these latter 2 monkeys, a CMLV also was recovered from the throat and/or salivary glands. Of the CMLV isolations made, 3 have been from monkeys born in the WRAIR colony, 2 adults and 1.5 month old infant. These findings not only indicated the high frequency of CMLV infection in the WRAIR Aotus colony, but also demonstrated spread of the virus among the monkeys.

Preliminary data indicated that CMLV persists for prolonged periods in the throat secretions of infected monkeys. With few exceptions, CMLV was reisolated from select monkeys 1, 2, 3, and 12 months after the initial isolation. Further studies are in progress to define the occurrence and persistence of CMLV in throat secretions, and have been extended to include the same parameters for blood and urine.

Of the 74 owl monkeys tested serologically, 65 (88%) had serum neutralizing antibodies against the reference CMLV 7933T isolate. CMLV were detected in the throat secretions of 82% (45/55) of the seropositive monkeys. CMLV also were recovered from throat swab specimens of 5 of 6 seronegative monkeys. Serum neutralization tests using the homologous isolate for 2 of these monkeys indicated the presence of antibodies against the recovered virus. These observations strongly suggested the existence of antigenic differences among the CMLV isolates. Studies have been initiated to compare the antigenic relationships of the different isolates and to develop suitable serological reagents to classify CMLV isolates.

The antigenic relationship between the CMLV isolates from owl monkeys and the human, rhesus and African green monkey strains of cytomegaloviruses was examined. Antigenic comparisons also were made for other herpesviruses recovered from Aotus monkeys and other primates, to include H simplex types 1 and 2, H simiae,

H. zooster, H. tamarinus, H. saimiri, H. ateles, H. aotus types 1, 2, and 3, Liverpool vervet agent, and herpes leukocyte-associated agent. None of the antisera against the cytomegaloviruses and other herpesviruses tested neutralized the CMLV isolates from the owl monkeys. Thus, the CMLV isolates appeared to be an antigenically distinct group of herpesviruses.

Further evidence of the distinctiveness of the Aotus CMLV from other human and monkey cytomegaloviruses was obtained from attempts to propagate the CMLV isolate in human and subhuman primate cell cultures. The CMLV isolate only could be propagated in cultures of Aotus cells. Cytopathic effects were not detectable in primary or continuous cultures of rhesus monkey kidney (LLCMK2), African green monkey (Vero), human lung (WI-38), or HeLa cells.

The third group of viruses isolated from owl monkeys in the WRAIR colony produced an adenovirus-type of CPE (5). Two of the isolates were recovered from rectal swab specimens from each of 2 adult owl monkeys, 1 of which was born in the WRAIR colony. The remaining isolates were obtained from the lungs, liver, spleen, and rectal swab specimen of a 5-month-old monkey that also had been born in the colony. Representative isolates from each monkey produced intranuclear inclusions in infected cell cultures, and suspensions of each fixed complement with reference adenovirus group antiserum. Electron microscopic examination of cell cultures infected with isolate 8138R showed the presence of typical adenoviruses in the cell nuclei. The isolates, therefore, were classified as adenoviruses. Additional studies will be made to determine the antigenic relationships among this group of isolates. These viruses appear to be quite prevalent among owl monkeys of the WRAIR colony. Serum neutralization titers of 1:4 or greater to the 8138R isolate were detected in 83% (39/47) of the monkeys tested.

Anemia and renal diseases are 2 of the principal health problems affecting monkeys in the WRAIR Aotus colony. In man and other animals, chronic viral infections often have been associated with these 2 disease syndromes, with the pathology observed being that of an immune complex disease rather than an acute viral infection. CMLV were isolated from almost 90% of the owl monkeys in the WRAIR colony, and reisolations from infected animals could be made for periods of at least 1 year. Further, these infected monkeys had serum neutralizing CMLV antibodies. Such observations are consistent with a chronic, persistent infection associated with an ineffective antibody response. In man, cytomegaloviruses often persist in the throats and urine

of infected individuals for considerable periods, even in the presence of neutralizing antibodies. Furthermore, cytomegalovirus infections in man have been associated with immune complex disease (6) and autoimmune hemolytic anemia (7). Mice experimentally infected with cytomegalovirus developed immune complex deposits in the glomeruli (8) and had markedly lowered resistance to infection with several microorganisms (9). By analogy, the CMV are prime candidates for inducing immune complex disease in owl monkeys, and could cause a decrease in resistance of the monkeys to infection with other microbes, such as Klebsiella pneumoniae.

The potential effect of adenovirus infection in the owl monkeys can not be overlooked, especially since 83% of the monkeys tested were seropositive for adenovirus antibodies. Like the cytomegaloviruses, adenoviruses have been associated with persistent infections. Such chronic infections in dogs have resulted in the deposition of adenovirus-antibody complexes in the kidneys (10).

Further elucidation of the role of the Aotus CMV or adenoviruses in the renal and anemia syndromes will be aided by the development of suitable tests for the viral antigens and antibodies. Once developed, the tests will be used to evaluate the pathogenic effects of deposition or circulation of viral antigens, antibodies, or their complexes.

4. Enzyme-linked immunosorbant assay for canine distemper virus.

Canine distemper virus (CDV) is a wide spread, highly infectious agent capable of causing disease in all breeds of dogs. Prospects for distemper and distemper-potentiated bacterial disease are especially menacing when large numbers of dogs from far flung environs are assembled into confined quarantine, holding, or breeding facilities. Such is the case when unconditioned random-source (RS) dogs are purchased from vendors. This problem is presumably obviated when properly conditioned dogs are purchased and assembled.

At the Walter Reed Army Institute of Research (WRAIR), both conditioned and unconditioned RS dogs have been purchased for research purposes. Each dog was tested for serum neutralizing (SN) antibodies to CDV. The results showed that 74% of the dogs which arrived without CDV antibody developed respiratory disease, and 32% died. In contrast, 24% of those with antibody developed respiratory disease, and only 4% died (11).

For these reasons, vaccination to protect against distemper becomes a necessity. However, even the purchase of conditioned dogs does not guarantee efficacious immunization, especially if the virus is incubating at the time of vaccination. The immune status of unconditioned (unvaccinated) is obviously worse. Consequently, it is very desirable to assess the immunity of all these dogs. At our installation one of the criteria for rejection is a SN-CDV antibody titer of less than 1:100.

Contractual agreements usually stipulate that there be a limited amount of time between dog delivery from a vendor and rejection by colony authorities. The SN test requires 3 days to perform and entails the need for tissue culture facilities. The enzyme-linked immunosorbant assay (ELISA) is a very simple and rapid seroassay which has been used to detect antibody to other viruses (12-17). Saunders observed > 95% correlation between the hog cholera virus ELISA results and the SN results (18). This report demonstrates the feasibility of using the ELISA test to measure the CDV immune status of dogs by correlating ELISA results with SN results, and describes the IgM and IgG response of dogs which have been experimentally infected.

Cells and viruses. The Ondestepoort strain of CDV was used for the ELISA and SN test, and was propagated in Vero cells using techniques described by Appel and Robson, with the following modifications (19). Cell monolayers of Vero cells, propagated in roller bottles (420 X 100 mm) were inoculated at confluency (approximately 2.4×10^6 cells) with 10 to 15 ml of CDV suspension which titered $10^{4.5}$ TCID₅₀ per ml. After 1 hour of adsorption at 36 C at a roller setting of 30 rph, 50 ml of fresh medium were added. The medium was replaced with serum-free medium 16 hours before harvesting. The serum-free medium was removed and discarded at 40 to 42 hours post-inoculation. The cell-associated virus was harvested by freeze-thawing the cells 3 times in 10 ml of serum-free medium. The suspension was sonicated for 30 seconds in an ice bath using the microtip on a Branson Mdl U108 Sonicator at #7 setting, and clarified at 300 X g for 15 minutes to remove cell debris. The supernatant was stored at -60 C before titration and use as CDV antigen. The protein concentration of the antigen used in the ELISA was as determined by the Lowry method (20).

Sera. Canine anti-CDV serum lot #d3 obtained from the US Department of Agriculture, Veterinary Biologics Laboratory, and National Animal Disease Center, Ames, IA, was used as

positive reference serum in all tests. Serum samples were obtained from random-source (RS) dogs at the time of their arrival at the WRAIR and stored at -20 C. Samples also were obtained from 2 dogs, 10 to 12 weeks old, experimentally infected with the mildly virulent a-76-30 field strain of CDV. Serum samples were collected at weekly intervals post-infection and stored at -20 C. Twenty-five serum samples of known CDV antibody titer also were obtained. The latter 2 groups of sera were kindly provided by Dr. Max Appel, Cornell University, Ithaca, NY.

Conjugates. Rabbit anti-dog IgG conjugated to horseradish peroxidase (Miles Laboratories) was used in the ELISA for detection of canine IgG. Goat anti-dog IgM conjugated to horseradish peroxidase (Cappel Laboratories, Inc) was used in the ELISA for detection of canine IgM. The conjugates were stored at -20 C and diluted immediately prior to use.

Plates. Polystyrene microelisa substrate plates (Dynatech, Inc) were used for all ELISA. Cell culture microtitration plates (Costar) were used for all SI tests.

Buffer and solutions. Carbonate coating buffer, pH 9.6, and phosphate-buffered saline-tween 20 buffer (PBST), pH 7.4, were prepared according to Voller (21). The overcoat solution was 4% (w/v) bovine serum albumin (BSA) in distilled water. The conjugate diluent was prepared by adding 1% (w/v) BSA to PBST buffer. The substrate solution of 5-aminosalicylic acid (5AS) with hydrogen peroxide was prepared according to Ruitenberg (22). Medium for the SI test was the same as that used to culture CDV.

ELISA procedure. The optimal antigen concentration was determined by a two-dimensional, checkerboard titration of antigen against serial, two-fold dilutions of USDA-positive reference serum and known negative serum. A 1:10 dilution of the CDV antigen containing 544 ug of protein per ml was the optimal dilution for distinguishing between positive and negative sera (22). The optimal conjugate concentration was determined by the same method. A 1:200 dilution of the anti-IgM conjugate and a 1:500 dilution of the anti-IgG conjugate were used.

The following sequential steps describe the microelisa procedure used throughout this study. An optimal concentration of antigen in carbonate buffer was added to each well of a substrate plate in 0.1 ml amounts and let dry overnight at

room temperature. Plates were washed 3 times, for 3 minutes each, with PBST buffer. Plates were either used immediately or stored in a humid chamber at 4 C. An overcoat of 0.1 ml of BSA was added to each well and incubated 30 minutes at 37 C. Sera were diluted with PBST buffer, 0.1 ml added per well, and then incubated 30 minutes at 37 C. Plates were washed again as described above. An optimal concentration of conjugate in PBST buffer with 1% BSA, was added in 0.1 ml amounts to each well and incubated 60 minutes at 37 C. Plates were again washed as described above. Substrate solution was added in 0.1 ml amounts to each well and incubated 1 hour at room temperature.

Control reactions for ELISA. Control reactions were performed in all experiments on the substrate, conjugate, and positive and negative sera (22).

Interpretation of ELISA results. The color reaction was read visually and scored 4+, 3+, 2+, 1+, -, according to the intensity of purple-brown color. A 1+ was interpreted as a positive reaction. Any serum which caused a positive reaction at a dilution $\geq 1:100$ was interpreted as positive for CDV antibody and was considered to be protective.

SN procedure. The SN test was performed as described by Appel and Robson (19).

Interpretation of SN results. Virus neutralizing antibody was interpreted as being absent in serum-virus mixtures if a single focus of viral CPE was observed on any monolayer by day 3 post-inoculation. For screening purposes, sera which showed virus neutralization at a dilution $\geq 1:100$ were interpreted as positive for protective antibody versus CDV.

Comparison of ELISA and SN test results. Two methods were used to compare results obtained by the ELISA and the SN test: (1) percentage of agreement (23) and (2) sensitivity and specificity (24).

Comparison of ELISA and SN. Sera were obtained from 221 random-source dogs immediately after their arrival at the WRAIR. Initially, 62 sera were diluted by two-fold serial dilutions from 1:25 to 1:3200. The anti-CDV activity of these sera was measured by ELISA and SN tests (Table 9). Sixty of the 62 (97%) yielded ELISA and SN endpoints which agreed within a four-fold difference. This small difference was assumed to be within experimental error limits and disregarded. There were

2 sera which were 8-fold lower in the ELISA test. Another noted characteristic was the 8 sera which had some activity at 1:25 and 1:50 dilutions in the ELISA but no discernible activity in the SN test at a 1:25 dilution. Three of these sera were still available and were retested at SN dilutions as low as 1:6.25. One serum showed some anti-viral activity, the other two did not. The reason for these differences is not readily discerned, but one should keep in mind that these 2 tests detect 2 very different phenomena. The SN test requires a complete and fully active virus and viricidal antibody, while the ELISA can detect whole CDV and sub-viral antigenic components. The ELISA has been shown to have equal or greater sensitivity as radioimmunoassay in some tests (25).

The remaining 159 sera were examined by a screening test based on the results obtained from the preceding 62 sera. Since antibody activity at \leq 1:100 dilutions in CDV-SN tests infers immunity to distemper, a comparable dilution also was used in the ELISA. Apparently a 1:100 titer in the ELISA also is indicative of immunity (Table 9).

A statistical analysis of both studies (221 sera) at a threshold dilution of 1:100 (Table 10) showed that there was 98% agreement. The ELISA had a sensitivity (ability to call a positive serum positive) of 96.5% and specificity (ability to call a negative serum negative) of 100%.

The discrepancy between the ELISA low titers versus an apparently complete lack of activity (Table 9) in SN tests was further investigated. We had suggested previously that different antigens and antibodies were involved. Our major problem was that we had no idea whether the RS-SN negative dogs had ever had distemper or been immunized with CDV. The only way to ascertain the complete lack of antibody was to obtain sera from dogs reared in a closed colony and with complete medical histories, documenting no immunization or infection with CDV. Twenty-five sera meeting these requirements were obtained from Dr. Max Appel. In a blind study which also contained positive sera, all the sera were tested by ELISA (negative = no reaction at 1:25; positive = reaction at 1:100) and SN (negative = no reaction at 1:12.5; positive = a reaction at 1:100). Nineteen of these sera were negative by both tests, 6 were positive by both tests. Thus, there was complete agreement between the ELISA and SN results.

Comparison of IgM and IgG response to CDV. Up to this point the ELISA was capable only of detecting canine IgG. When the

conjugated goat anti-canine IgM became available, it was possible to measure both of these immune globulins and compare their titers to those of the SI test. Two dogs (A and B) which were experimentally infected with CDV were serially bled after inoculation of the virus. The SN titers of both dogs decreased 2- to 4- fold around the third week of infection. This coincided with a drop in the IgM of these sera (Fig. 2). At the next interval the increase in SN titer coincided with the increase in specific IgG. It appears that the early part of the SN test profile is made up of IgM viricidal antibody and the later part of the SN is made up of viricidal IgG antibody.

One further point should be emphasized, it is not unusual to use an anti-IgG reagent to monitor immune responses. We had previously used it to monitor antibody response to ICH (5) where the ELISA-IgG titers very closely approximated SI titers.

5. Recovery of a canine coronavirus from laboratory dogs with fatal diarrhea.

Coronaviruses are important etiologic agents of severe and often fatal diarrhea in neonatal swine, cattle, mice and turkeys. A canine coronavirus also has been associated with diarrheal disease in military dogs (5,26) and has been found in the intestinal tracts of laboratory dogs with fatal, multiple viral infections (11). During the first quarter of this year, informal reports linked this virus with severe, and at times fatal, diarrheal disease in dogs (News, JAVMA 173: 247-248, 1 Aug 1978). An outbreak of severe diarrheal disease occurred at the WRAIR animal facilities in March among a litter of 10 week old Foxhound dogs with 2 fatalities. This outbreak afforded an opportunity to study the etiology of diarrhea in laboratory dogs which could be examined in greater detail than in privately owned dogs. This report summarizes the clinical observations and etiologic studies, and provides further evidence for the etiologic significance of coronavirus infections in canine diarrhea.

Seven 8-week-old Foxhound dogs were received from a licensed animal vendor in mid-March 1978. Prior to arrival at the WRAIR, the dogs had been treated 4 times with Task at 2-week intervals for intestinal parasites and had been given 2 doses of canine distemper (CD)-infectious canine hepatitis (ICH)-leptospira vaccine. The dogs were given 1 dose of CD-ICH-canine parainfluenza (CPI)-leptospira vaccine on the day of arrival at the WRAIR followed by Vermiplex for intestinal parasites 5 days later.

Signs of disease were first noticed 10 days after arrival when 5 of the 7 dogs developed diarrhea. The following morning 1 of the affected dogs was found dead and the other 2 dogs in the same cage appeared severely ill. These latter 2 dogs had severe diarrhea, were vomiting, and were markedly dehydrated and lethargic. Both dogs were given supportive treatment to replace the loss of fluids, drugs to control the diarrhea and vomiting, and antimicrobial therapy; however, one dog died. The remaining dog began to improve the following day and was completely recovered on the 9th day. One of the 2 other sick dogs developed severe diarrhea on the fourth day of illness, was given similar supportive therapy, and recovered. Two dogs did not develop any signs of disease.

Specimens for bacterial and viral studies were obtained from both the affected and apparently normal dogs. Also, tissues were obtained at necropsy from the 2 dead dogs. The procedures for bacterial and viral studies have been described in detail.

Salmonella sp or other known canine bacterial enteric pathogens were not recovered from rectal swabs or the intestinal tract tissues. Several isolations of E coli were made and the isolates being tested for cellular adhesiveness and toxigenicity, although the role of pathogenic E coli in canine diarrhea disease is unknown. Canine coronaviruses were isolated from 4 of the 5 sick dogs and from both apparently healthy dogs. The coronaviruses were recovered in high titer from intestinal tissue of both dogs that died (10^{-4} and 10^{-5} /gm, respectively). In addition, canine parainfluenza (CPI) virus was recovered from the lungs of 1 of these dogs. The dogs had received vaccine containing attenuated CPI vaccine 11 days earlier and the origin of the CPI isolate could not be ascertained.

Serologic tests confirmed the virus isolation tests. All 5 surviving dogs developed neutralizing antibody to both the isolated coronavirus and the 1-71 reference canine coronavirus. The dogs also developed neutralizing antibody to ICH and CPI viruses. Although all the dogs had high antibody titers to CD virus in their acute serum specimens, 3 dogs developed a further 4-fold increase in titer to this virus. The response to the latter 3 viruses were consistent with their vaccination histories.

The findings in this study provide further evidence for the significance of canine coronaviruses in enteric disease of the dog. The virus was present in the feces and intestinal tracts in high titer of dogs with severe and fatal diarrhea. The

serologic findings confirmed the virus isolation test results as each of the surviving dogs developed neutralizing antibody. Further studies are required to determine significance of bacteria (e.g. E coli), parasites, or other agents in the intestine which may potentiate the severity of the coronavirus infection.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 011 Health Care and Management of Laboratory Animals

Literature Cited.

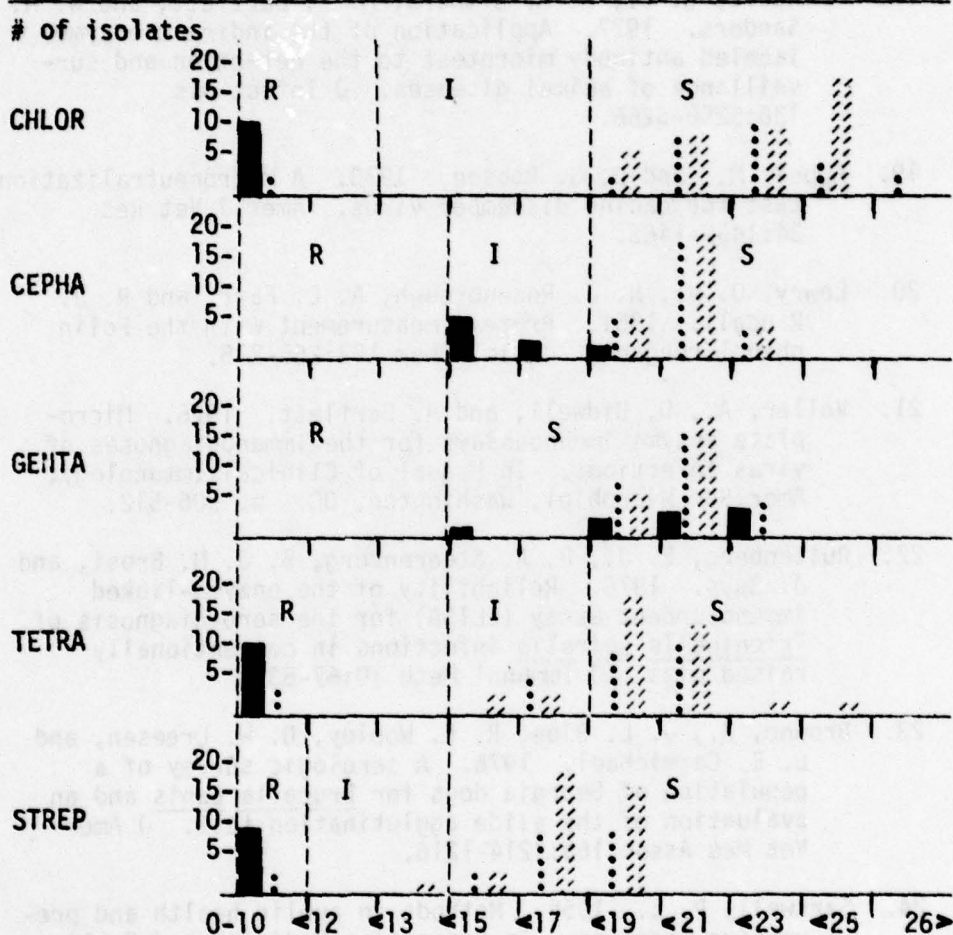
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Figure 1. Relationship of Serotype 2, Indol + and Indol - Strains of *K pneumoniae* Isolated From Aotus Monkeys and Their Resistance to Selected Antibiotics as Measured by Zone Size of Inhibition



CHLOR = chloramphenicol
 CEPHA = cephalothin
 GENTA = gentamicin
 TETRA = tetracycline
 STREP = streptomycin
 R = resistant

I = intermediate
 S = sensitive
 TYPE 2 //
 INDOL +
 INDOL -, untypeable

Figure 2. Comparison of SN Antibody Titers, ELISA IgM and IgG Antibody Titers from 2 Dogs Experimentally Infected with CDV

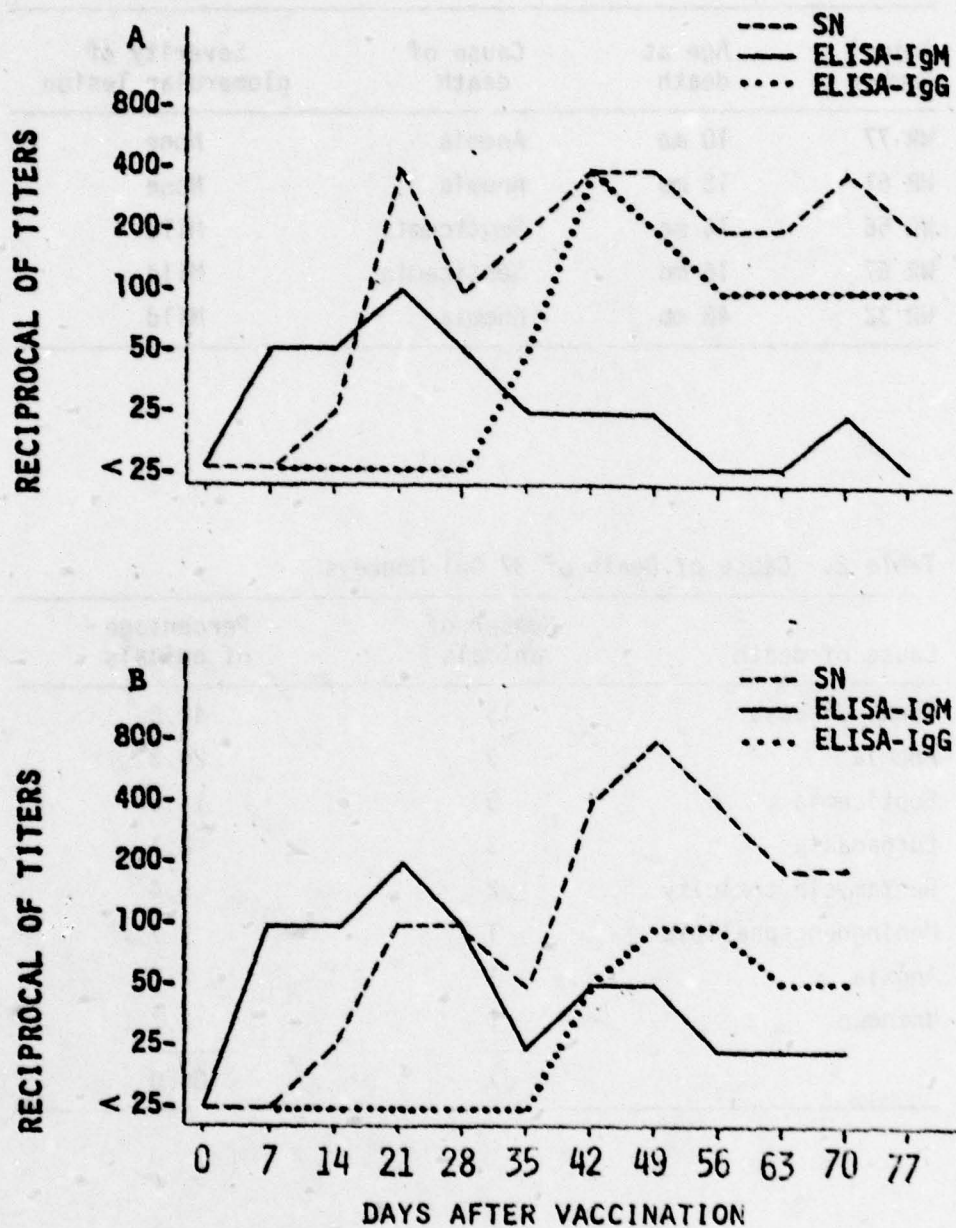


Table 1. Age, Cause of Death, and Severity of Glomerular Lesions of 5 Owl Monkeys Born at the WRAIR

Animal number	Age at death	Cause of death	Severity of glomerular lesion
WR 77	10 mo	Anemia	None
WR 61	15 mo	Anemia	None
WR 56	16 mo	Septicemia	Mild
WR 67	16 mo	Septicemia	Mild
WR 32	48 mo	Anemia	Mild

Table 2. Cause of Death of 37 Owl Monkeys

Cause of death	Number of animals	Percentage of animals
Renal disease	15	40.6
Anemia	9	24.3
Septicemia	5	13.5
Euthanasia	3	8.1
Gentamycin toxicity	2	5.4
Meningoencephalitis	1	2.7
Anoxia	1	2.7
Unknown	1	2.7
	37	100.0

Table 3. Histopathological Lesions Most Frequently Observed in 37 Owl Monkeys

Lesion	Animals affected	Animals examined	% of animals examined
Glomerulonephritis	34	37	91.9
Glomerular fibrosis	24	37	64.9
Interstitial nephritis	31	37	83.8
Hypercellular bone marrow	30	33	90.9
Splenic R.E. hyperplasia	28	32*	87.5
Splenic erythrophagocytosis	24	32	75.0
Extramedullary hematopoiesis	21	37	56.8
Myocardial fibrosis	22	37	59.5
Myocarditis	15	37	40.5
Atherosclerosis	13	37	35.1
Perivasculitis	9	37	24.3
Interstitial pneumonia	14	37	37.8

*Spleens were not present at necropsy in 5 monkeys.

Table 4. Comparison of the Severity of Glomerulonephritis with the Cause of Death

Severity of GN	Cause of Death			
	Anemia	Renal disease	Septicemia	Others
Severe	2	9	0	1
Moderate	3	4	1	5
Mild	2	2	4	1
None	2	0	0	1
	<u>9</u>	<u>15</u>	<u>5</u>	<u>8</u>

Table 5. Comparisons of Typical Antibidiograms for Klebsiella pneumoniae Isolates from Aotus Monkeys

<u>Klebsiella pneumoniae</u>		<u>Antibiotic sensitivities^a</u>									
Serotype	Biotype	AMP	CB	CF	C	GM	ST	SXT	F	Te	G
Type 2	Indole Neg	R	R	S	S	S	S	S	S	S	S
Non Type 2	Indole Neg	R	R	S	S	S	S	S	S	S	S
Non Type 2	Indole Pos	R	R	I	R	S	R	S	S	R	R
Non Type 2 (Isolate 27-10-27)	Indole Neg	R	R	I	R	S	R	S	S	R	R

^aAbbreviations:

AMP = Ampicillin
 CB = Carbenicillin
 CF = Cephalothin
 C = Chloramphenicol
 GM = Gentamycin
 ST = Streptomycin
 SXT = Trimethoprim

F = Furadantoin
 Te = Tetracycline
 G = Gantrisin (sulfa)
 R = Resistant
 S = Sensitive
 I = Intermediate

Table 6. Antibidiograms of *Klebsiella pneumoniae* Indole Biotypes from the Monkey Colony Environment

Source	Indole production	Antibiotic sensitivities ^a									
		AMP	CF	C	Gm	ST	SXT	F	Te	G	
Orange	+	R	S	S	S	S	S	S	S	S	
Orange	+	R	S	S	S	S	S	S	S	S	
Orange	+	R	S	S	S	S	S	S	S	S	
Orange	-	R	S	S	S	S	S	S	S	S	
Orange	-	R	S	S	S	S	S	S	S	S	
Orange	-	R	S	S	S	S	S	S	S	S	
Monkey chow	-	R	S	S	S	S	S	S	S	S	
Pet cal	-	R	S	S	S	S	S	S	S	S	
Roach	-	R	S	S	S	S	S	S	S	S	
Water bowl	-	R	S	S	S	S	S	S	S	S	

^aAbbreviations:

AMP = Ampicillin
 CF = Cephalothin
 C = Chloramphenicol
 Gm = Gentamycin
 ST = Streptomycin
 SXT = Trimethoprim

F = Furadantoin
 Te = Tetracycline
 G = Gantrisin (sulfa)
 R = Resistant
 S = Sensitive

Table 7. Average Values for Select Clinical Pathology Parameters for Three Distinct Groups of Aotus Monkeys

Group ^a	Number monkeys	Date examined and parameter								
		September 1977				March 1978				
		RBC ^b X10 ⁶	Alb ^c g/dl	Bun ^d mg/dl	Cr ^e mg/dl	RBC X10 ⁶	Alb g/dl	Bun mg/dl	Cr mg/dl	
1	3	5.00	4.1	24.6	0.59	5.06	3.6	18.5	0.65	
2	13	5.06	4.3	21.4	0.64	5.30	4.0	18.4	0.69	
3	10	4.91	4.5	23.3	0.75	5.16	3.9	22.4	0.62	

^aGroup 1 consisted of monkeys that were chronically colonized with Klebsiella pneumoniae type 2; Group 2 was comprised of monkeys yielding sporadic isolations of Klebsiella sp. and Group 3 were monkeys from which Klebsiella sp could not be isolated.

^bErythrocytes X 10⁶/cmm of blood

^cAlbumin

^dBlood urea nitrogen

^eCreatine

Table 8. Recovery of Cytomegalo-like Viruses from Aotus Monkeys

Monkeys examined	Specimen examined	Number positive Total examined	% Pos
Adult, dead	Throat	6/21	29
	Air sac	0/18	0
	Salivary glands	17/33	52
	Lungs	0/40	0
	Liver	0/40	0
	Kidney	0/40	0
	Spleen	1/40	3
	Rectal	0/23	0
Fetal and infant, dead	Throat	0/3	0
	Air sac	1/7	14
	Salivary glands	1/11	9
	Lungs	0/15	0
	Liver	0/15	0
	Kidney	0/15	0
	Spleen	0/15	0
Adult, apparently healthy	Throat	37/43	86
	Rectal	0/8	0
	WBC	1/8	13
	Urine	0/6	0
Infant, apparently healthy	Throat	0/3	0
	Rectal	0/3	0

Table 9. Titers of 62 Sera Assayed for CDV Antibody.
Comparison of ELISA and Serum Neutralization Tests.

SN titer	Number of sera with indicated ELISA titer:								total
	< 25	25	50	100	200	400	800	1600	
< 25	1	3	5						9
25			1						1
50		2	4	1					7
100				4					4
200			1	3	3		1		8
400				1	7	5			13
800					4	5	3		12
1600						2	1		3
3200						2	1	2	5

Table 10. Two Methods for Comparing the CDV ELISA With the CDV Serum Neutralization Tests.*

Method 1. Percentage of agreement:

<u>No. sera tested</u>	<u>ELISA</u>	<u>SN</u>	<u>Results</u>
107	107-	107-	
110	110+	110+	217 agree
4	4-	4+	4 disagree
0	0+	0-	221 total

$$\text{Percentage of agreement} = \frac{217}{221} = 98.2\%$$

Method 2. Sensitivity and specificity of a test:**

<u>ELISA</u>	<u>SN test</u>		
	<u>positive</u>	<u>negative</u>	<u>total</u>
110+	(A) 110	(B) 0	110
111-	(C) 4	(D) 107	111
221	(A+C) 114	(B+D) 107	221

$$\text{Sensitivity} = \frac{A}{A+C} = \frac{110}{114} = 96.5\%$$

$$\text{Specificity} = \frac{D}{B+D} = \frac{107}{107} = 100\%$$

* In both methods of comparison the SN test is considered standard.

** Sensitivity is defined as the ability to declare a positive serum positive. Specificity is defined as the ability to declare a negative serum negative. (A) = no. sera positive in SN and positive in ELISA. (B) = no. sera negative in SN and positive in ELISA. (C) = no. sera positive in SN and negative in ELISA. (D) = no. sera negative in SN and negative in ELISA.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OB 6444	78 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISSEM INSTN ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
a. PRIMARY -	62770A	3M162770A802	00	012			
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Diseases of Military Dogs							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
010100 Microbiology 005900 Environmental Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
68 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATES/EFFECTIVE: NA				PRECEDENCE		b. FUNDS (in thousands)	
b. NUMBER: NA				FISCAL YEAR		2.5	
c. TYPE:				CURRENT		300	
d. AMOUNT:				79		1.5	
e. CUM. AMT.				79		90	
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
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24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number, precede text of each with Security Classification Code)							
<p>23. (U) To investigate diseases and/or conditions affecting or associated with the military dog to enhance diagnosis, treatment, and control.</p> <p>24. (U) Conventional epidemiologic, pathologic, and microbiologic methods are employed; special procedures are developed as needed.</p> <p>25. (U) 77 10 - 78 09 The susceptibility of somatic cell hybrids between canine peritoneal macrophages and SV40-transformed human cells was correlated to the phagocytic capability of the cell population. Phagocytosis, however, was apparently under a repressor control mechanism and a population of cells exhibiting this characteristic and total susceptibility to Ehrlichia canis infection was unattainable. Canine peritoneal macrophages could not be transformed by direct infection with SV40 to provide a continuous culture of canine macrophages to support propagation of E. canis. The prevalence of ehrlichial antibodies among military dogs from USA and USAF installations worldwide was greatest in dog sera from SE Asia. A canine cell line that had been previously derived from a canine tumor was characterized morphologically, karyotypically, and enzymatically. The cell is susceptible to infection with most canine viruses, and is the cell of choice for canine coronaviruses, causative agents of viral diarrhea. An epizootic of respiratory disease among military dogs at Lackland AFB, TX, in Dec 77 was attributed to canine parainfluenza SV5. Two of the affected dogs had been vaccinated against the virus, indicating the necessity for further surveillance and evaluation of the efficacy of the SV5 vaccine. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.</p>							

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Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 012 Diseases of the Military Dog

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Description:

To define, study, diagnose, and control known and potential infectious diseases of military dogs. A major effort is directed toward developing an alternate in vitro method of cultivating Ehrlichia canis in lieu of the primary canine peripheral blood monocyte or peritoneal macrophage. Additional studies concern the epizootiology, diagnosis, treatment, and control of other disease agents or conditions affecting the military dog.

During the reporting period, research activities have included: (1) attempts at cultivation of E. canis in cells other than primary canine peripheral blood monocytes or peritoneal macrophages, (2) characterization of a canine cell line for propagation of canine coronaviruses, and (3) serologic evaluation of an epizootic of respiratory disease among US military dogs at Lackland AFB, Texas.

1. Somatic cell hybrids of canine peritoneal macrophages and SV-40 transformed human cells: Use for propagation of Ehrlichia canis.

Canine ehrlichiosis continues to be a significant disease problem among military dogs, especially those deployed in tropical and subtropical areas (1). Continued productive research on ehrlichiae and the disease they cause, however, has been impeded by an inability to propagate the agent in large numbers and the absence of a reproducible method for quantification.

Recently, a somatic hybrid cell was derived from the fusion of canine peritoneal macrophages and SV40-transformed human cells (2). This hybrid cell showed a definite potential of being capable of supporting Ehrlichia canis replication, as a pro-

portion of the cell population could be infected and an increase in the size and number of cytoplasmic inclusions occurred. The purposes of this study were : (1) to define the characteristics of the E canis-susceptible cell population as contrasted with the non-susceptible population and (2) to obtain clones of somatic hybrid cells that were totally capable of supporting ehrlichial growth.

Cells. Somatic hybrid (WRH-2) cells were obtained from the fusion of canine peritoneal macrophages and SV40-transformed human skin fibroblasts (2), and were maintained in hypoxanthine-aminopterin-thymidine (HAT) medium (3). Primary cultures of canine peripheral blood monocytes were prepared as previously described (4).

Determination of phagocytosis capability. Cultures of WRH-2 cells were prepared by seeding 2.5×10^5 cells into Leighton culture tubes containing 9 X 35 mm glass coverslips, which yielded a 60 to 70% monolayer after 48 hr incubation at 37 C. The culture medium was Eagle's minimal essential medium in Earle's balanced salt solution (EMEM) supplemented with 1 mli glutamine, 10 mM each of nonessential amino acids (NEAA), and 10% fetal calf serum (FCS). Canine peripheral blood monocyte cultures were prepared as previously described (4). Stock suspensions of Candida albicans were prepared by growing the yeast in trypticase soy broth for 48 hr at 25 C. Yeast cells were pelleted by centrifugation at $6,000 \times g$ for 15 min at 4 C, then washed 3 times in 0.85% NaCl using the same centrifugation conditions. The final yeast pellet was resuspended in 0.85% NaCl to a final concentration of 1×10^8 yeast cells/ml and the yeast cells were heat inactivated at 100 C for 30 min. Stock yeast suspensions were stored at -20 C until used. For phagocytosis determinations, the stock yeast suspension was diluted in the appropriate culture medium to a concentration of 7.5×10^6 yeast cells/ml. Culture medium was removed from Leighton tube cultures and 1 ml of yeast-containing culture medium was added, which provided a culture cell to yeast cell ratio of approximately 1:15. Cultures were incubated for 24 hr at 37 C. Coverslips were washed 3 times with phosphate buffered saline, extracted, and stained using the Giemsa method. The relative percentage of cells containing yeasts was determined by microscopic examination of 500 cells.

Separation of phagocytic and non-phagocytic cells with carbonyl iron. WRH-2 cells were propagated in 32 oz bottles to obtain confluent monolayers. To each culture bottle, an aliquot of 5 mg of sterile carbonyl iron was added, and the culture was

incubated at 37 C for 2 hr. Cells were dispersed with trypsin-EDTA and pelleted by centrifugation at 900 X g for 10 min at 4 C. The cell pellet was resuspended in 25 ml of Hanks' balanced salt solution (HBSS). The suspension was exposed to a magnet to sediment the carbonyl iron-containing cells and free iron particles. After aspiration of all but 1 ml of the non-sedimented cell suspension the extraction procedure was repeated twice more. The final cell pellet was used to seed Leighton culture tubes and 25 cm² flasks at 2.5 X 10⁵ cells/ml in HAT + 10% FCS. Leighton tube cultures were used for phagocytosis determinations and 25 cm² flasks were used for cloning.

Cloning of WRH-2 cells. WRH-2 cells were cloned using the glass chip method. Each of 4 - 60 mm petri dishes containing 50 to 100 glass chips (0.5 to 1.0 mm diameter) was seeded with 7.5 X 10³, 1.0 X 10⁴, 1.5 X 10⁴, and 2.0 X 10⁴ cells in a total volume of 5 ml of culture medium. Cultures were incubated for 18 to 24 hr at 37 C in 10% CO₂ to obtain attachment and spreading of the cells. Using an inverted microscope and forceps, glass chips with only 1 cell attached were retrieved and transferred to an individual well of a 24-well culture dish, each well of which contained 1 ml of culture medium. Cultures were incubated in a 10% CO₂ atmosphere at 37 C until individual clones formed.

Infection of cells with Ehrlichia canis. Cultures of cells in Leighton culture tubes were prepared as described above. Inocula consisted of E. canis that had been propagated in primary canine peripheral blood monocytes. Growth medium was removed from the cell cultures, and 0.4 ml of inoculum was added to each Leighton tube culture. After a 2 to 3 hr adsorption period, the excess inoculum was removed and the cultures were fed with maintenance medium. The maintenance medium for WRH-2 cells was composed of EMEM that contained 1 mM glutamine, 10 mM each of NEAA, 5% FCS, and 0.5 ug of cycloheximide/ml, while medium for canine peripheral blood monocytes was EMEM supplemented with 20% canine serum. Inoculated cultures were incubated at 35 C. Maintenance medium was changed 24 hr after inoculation and every 3 to 4 days thereafter. Growth of E. canis in cells was monitored by staining the coverslip cultures using the direct fluorescent antibody (FA) technique at selected time intervals after infection.

WRH-2 cells were permissive for the replication of E. canis; however, only a small percentage, approximately 5 to 7%, possessed this characteristic. Infected cells were observed 5 days after infection, using the direct FA technique, and

contained 1 or more small inclusions or morulae. After 10 days, there was an increase in the size and number of morulae with a concomitant slight increase in the number of infected cells.

The percentage of hybrid cells permissive to E canis paralleled the proportion of the cell population that exhibited phagocytic properties for heat-inactivated Candida albicans. Subsequent selection for phagocytic cells using the carbonyl iron technique resulted in an increase of the phagocytic population to 50%. Cloning of these cells, however, did not yield a subpopulation of cells that was totally susceptible to ehrlichial infection. A fraction of each subclone cell population, which ranged from 2 to 25%, was phagocytic and could be infected with E canis. Further, the original enhanced phagocytic hybrid cell population and each of the subclone populations reverted to the 5 to 7% phagocytic level after 2 subpassages of the cells.

The concept of obtaining somatic cell hybrids between canine peritoneal macrophages and SV40-transformed human fibroblasts for the propagation of E canis arose from the production of similar somatic hybrid cells using mouse peritoneal macrophages (5) and the subsequent infection of the mouse-human hybrids with lactic dehydrogenase virus (6). In vitro replication of E canis, like lactic dehydrogenase virus, is limited to peritoneal macrophages and peripheral blood monocytes (4,7). Further, the limitation for E canis is species specific.

Somatic cell hybrids were derived from the fusion of primary canine peritoneal macrophages and SV40-transformed human skin fibroblasts, but the segregation pattern of the chromosomes was not as expected. It was anticipated that there would be preferential loss of human chromosomes from the hybrid cells, similar to the segregation pattern that was observed when mouse peritoneal macrophages and SV40-transformed Lesch-Nyhan cells were fused (5). The loss of canine chromosomes in the WRH-2 cells paralleled the observations reported for somatic hybrids of mouse peritoneal macrophages and HIT-1080 human fibrosarcoma cells (8).

The permissiveness of WRH-2 cells for E canis infection was associated with expression of phagocytic properties by select hybrid cells. Only 5 to 7% of the cell population expressed this property, and the percentage remained relatively constant through 60 cell passages. Further, significant enhancement of the phagocytic cell population could not be maintained after subcloning. These results suggested that the phagocytic

activity in most of the hybrid cells, and concomitant permissiveness to E canis infection, was being suppressed by some undetermined mechanism, presumably of the repressor type.

The inability to obtain a subclone of WRH-2 cells that was totally permissive to ehrlichiae precluded the continued use of the cell line for E canis investigations. However, since canine chromosomes were preferentially lost in the WRH-2 cells, these somatic hybrids could be used for the assignment of genes to specific canine chromosomes. Additional studies are in progress to explore the potential of WRH-2 cells in the propagation of other canine obligate microorganisms, such as canine herpesvirus and canine distemper virus.

The susceptibility of somatic cell hybrids (WRH-2 cells) between canine peritoneal macrophages and SV40-transformed human cells was correlated to the phagocytic capability of the cell population. Phagocytosis, however, was apparently under a repressor control mechanism and a population of cells exhibiting this characteristic and total susceptibility to E canis infection was unattainable.

2. Infection and transformation of canine peritoneal macrophages by SV40.

The only cultured cells that have been shown to be totally permissive for infection with Ehrlichia canis are primary cultures of canine peripheral blood monocytes (7) and peritoneal macrophages (4). Neither of these cell types, however, can be serially propagated and neither forms confluent monolayers. As a consequence, ehrlichiae can not be propagated in large numbers and a reproducible method for quantification can not be developed, two criteria necessary for continued productive ehrlichial research.

Mouse peritoneal macrophages have been infected and transformed by SV40 (9). Subclones of the transformed mouse macrophages had several characteristics of primary macrophages: high acid phosphatase and phagocytic activities, lysozyme production, and specific antigenic determinants. The retention of these characteristics by transformed mouse macrophages suggested a similar cell line might be obtained from primary canine peritoneal macrophages. Development of a SV40-transformed canine peritoneal macrophage cell line appeared to be a potential source of cells that would grow indefinitely in culture and would be permissive to infection with E canis, especially since permissiveness had been associated with cell

phagocytosis capability. The purpose of this study was to investigate the feasibility of employing virus transformation procedures with canine peritoneal macrophages.

Cells. Primary canine peritoneal macrophages were obtained by peritoneal lavage and used to seed 25 cm² plastic flasks as previously described (4).

Virus. SV40 was kindly supplied by Dr. B. Hampar (National Cancer Institute, Ft. Detrick, MD). The stock virus preparation contained 10⁸ PFU/ml in Eagle's minimal essential medium containing 1 mM glutamine and 3% fetal calf serum.

Conditioned medium. Conditioned medium was prepared by propagating canine peritoneal fibroblasts in RPMI 1640 containing 20% canine serum. After the cultures had been incubated at 37 C for 7 days, the culture medium was collected and filtered using a 0.2 micron filter to remove cell debris.

Infection of cells with SV40. Tissue culture medium was removed from flask cultures of peritoneal macrophages. One ml of stock SV40 suspension was added. After a 2 hr adsorption period at 37 C, excess inoculum was removed and 5 ml of maintenance medium was added. Maintenance medium was composed of RPMI 1640 containing 20% canine serum and 10% conditioned medium. Inoculated cultures were incubated at 35 C. Maintenance medium was changed 24 hrs after inoculation and every 3 to 4 days thereafter. Five 25 cm² flask cultures each were inoculated at 48, 72, and 96 hrs after initial seeding, and 5 uninoculated flask cultures were used as controls.

Subculturing of cells. Confluent monolayers of peritoneal macrophages, inoculated and uninoculated, were dispersed with trypsin-EDTA and split 1:2 into 25 cm² flasks containing 5 ml of maintenance medium.

Tumor (T) antigen and viral capsid (V) antigen determination. To determine the presence of T or V antigens, 1 ml aliquots of culture medium were removed from flask cultures and transferred to 15 X 45 mm glass vials that contained circular coverslips. Cells in the culture medium were deposited onto the coverslip by centrifugation at 900 x g for 20 min at 4 C. The coverslip was removed, air dried, fixed in cold acetone for 10 min, and stored at -20 C until used. Examination for T and V antigens was made using the indirect FA technique (these examinations were kindly done by Dr. B. Hampar).

The peritoneal macrophage cultures were approximately 80% monolayered when inoculated with SV40 at each of the time intervals. Confluent monolayers of inoculated cells were obtained by days 10, 20, and 30 after infection, at which times the cultures were subcultured. The relative proportion of the culture surface with attached cells in control flasks was slightly less than observed for the inoculated cultures at each of these time intervals. Three to 4 days following the third subculture at 30 days after inoculation, essentially all of the cells in each of the inoculated and control flasks detached. Greater than 95% of the detached cells were dead, as determined by the trypan blue exclusion test.

Examination of cell preparations made at each time interval the cultures were refed failed to demonstrate the presence of T or V antigens of SV40.

Canine peritoneal macrophages, unlike mouse peritoneal macrophages (9), were not permissive for SV40 infection. Consequently, transformation of the cells was unattainable with intact SV40. The apparent replication observed undoubtedly was the result of proliferation stimulation by macrophage growth factor in the conditioned medium, as has been reported for mouse peritoneal macrophages (10).

Canine peritoneal macrophages were not permissive for infection by intact SV40. In the absence of viral infection, transformation of the macrophages was unattainable.

3. Establishment of a canine cell line: derivation, characterization, and viral spectrum.

Primary and subcultivable cell cultures have been used extensively as a basic research tool in viral studies. Studies of canine viruses and the diseases they cause commonly have been accomplished using primary cultures of cells due to the limited number of available canine cell lines, such as Madin-Darby canine kidney cells and Cf2Th canine thymus cells (11).

Investigations were initiated to establish one or more new canine cell lines that could be employed for initial isolation, propagation, and study of canine viruses. Availability of additional cell lines would allow viral diseases of the canine to be elucidated more readily and extensively without reliance on continuous preparation of primary cell cultures, which is both expensive and time consuming. The purposes of this study were to: (1) define and characterize a canine cell line that

was established and (2) delineate the susceptibility of the cell line to infection with canine viruses.

Initiation of cell line. Three tumor nodules, each approximately 1 cm in diameter, were aseptically removed from an 8-year-old, female Golden Retriever dog. One nodule excised from the right inguinal mammary area and one from the medial aspect of the right stifle were processed for histopathologic examination. The third nodule, removed from the medial aspect of the left thigh was used for cell culture initiation. The tissue was minced into pieces of approximately 3 mm³ and washed twice with Hanks' balanced salt solution (HBSS). Seven pieces of tissue were transferred to each of 3 - 25 cm² plastic flasks and positioned at different locations in the flask. The flask with tissue pieces was incubated at 35 C for 1 hour to allow the tissue to adhere firmly to the surface, after which time 1.5 ml of growth medium was added to each flask. Growth medium was composed of Medium L-15 containing 1 mM glutamine, 30% fetal calf serum (FCS), 100 units of penicillin/ml, 100 ug of streptomycin/ml, and 2.5 ug of amphotericin B/ml. Cultures were incubated at 35 C.

Subculture methods. After the initial monolayers of cells were obtained, the cells were subcultured by dispersing with 0.05% trypsin - 0.02% ethylenediaminetetraacetate (trypsin-EDTA), pelleted by centrifugation at 900 X g for 10 min at 4 C, and splitting into fresh growth medium. Subsequently, cells were subcultured by splitting 1:3 and incubating at 37 C.

Morphologic characteristics. Cultures for cytologic examination were prepared by seeding 2.0×10^5 cells into Leighton culture tubes that contained 9 X 35 mm glass coverslips. At select intervals after incubation at 37 C, the coverslip cultures were washed twice with phosphate buffered saline, extracted, fixed in methanol, and stained by the Giemsa method.

Growth characteristics. Cells were seeded at a concentration of 1.1×10^6 cells into 75 cm² plastic culture flasks containing 30 ml of growth medium, and incubated at 37 C. At 6, 12, and 24 hr after seeding, and at 24 hr intervals thereafter, the cells in each of 3 culture flasks were dispersed with trypsin EDTA and counted in triplicate using trypan blue exclusion to indicate cell viability.

Karyotypic analysis. Giemsa (G-) banding staining of the metaphase chromosomes of the cells in the 128th passage level was performed by Lavappa's modification of the method described

by Seabright (12,13). Micrographs were made and analyzed of a minimum of 50 metaphases.

Enzyme analysis. Three strains of cultured cells were used as a source of enzymes: initiated cell line, canine kidney, and canine peritoneum. Cultures of canine kidney cells and canine peritoneum cells were prepared by the method of Holper (14). Canine kidney cells were propagated in Eagle's basal medium in Earle's balanced salt solution supplemented with 1 mM glutamine and 10% FCS, while canine peritoneum cells were grown in RPMI 1640 medium containing 10% FCS. Cells were adjusted to a concentration of 8×10^7 cells/ml and disrupted by homogenization or multiple freeze-thaw cycles in 1:20 Tris-EDTA-borate buffer, pH 8.6. Membranes and other particulate components were pelleted by centrifugation at 40,000 X g for 60 min. Supernatant enzymes were examined by vertical starch gel electrophoresis as described by Nichols and Ruddie (15). Staining of gels for specific enzymes was accomplished using minor modifications of established methods (15,16). Enzymes analyzed included glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1. 49), glucose phosphate isomerase (GPI, EC 5.3.1.9), lactate dehydrogenase (LDH, EC 1.1.1.27), mannose phosphate isomerase (MPI, EC 5.3.1.8), phosphoglucomutase (PGM, EC 2.7.5.1), and purine nucleoside phosphorylase (NP, EC 2.4.2.1).

Virus host range. Viral seed suspensions were propagated and titrated in the appropriate reference cells as shown (Table 1). Cultures of the initiated cell line were prepared by seeding 2.5×10^5 cells into 16 X 125 mm screw-cap culture tubes containing 1 ml of growth medium. After 48 hr incubation at 37 C, each of 2 culture tubes was inoculated with 0.1 ml of ten-fold dilutions of virus in maintenance medium, then incubated at 37 C for 1 hr for adsorption. The cultures were fed with 1.5 ml of maintenance medium, which was comprised of Medium L-15 supplemented with 1 mM glutamine, 10% FCS, and antibiotics. Inoculated cultures were incubated at 37 C. Tubes were examined daily for 6 days, with the degree of CPE being recorded. Hemadsorption was performed by standard techniques.

The tumor nodule removed from the right inguinal mammary area was diagnosed histopathologically as an adenocarcinoma. Diagnosis of the type of tumor from which the cell line originated, however, was not made due to the limited amount of tissue available.

The initial cell cultures attained confluency by 28 days after initiation, and consisted of fibroblast-like cells. These cells, which were designated A-72 cells, have been maintained in monolayer culture for more than 135 serial passages. Morphologic characteristics of A-72 cells have remained typically fibroblastic. The cells have vacuolated cytoplasm and spherical to ovoid nuclei containing several nucleoli.

Once established, monolayer subcultures grew to confluency in 6 to 7 days after a 3:1 split. The growth curve of A-72 cells in monolayer culture at the 123rd passage level showed a logarithmic increase in the number of cells between 6 and 120 hr after seeding (Fig. 1), with a population doubling time estimated to be 19 hr. A-72 cells did display density dependent inhibition (contact inhibition of cell division), and grew to a maximum density of 2.5×10^5 cells/cm².

Chromosome analysis of A-72 cells was made at the 128th passage level. The chromosome number, determined from counts on 30 metaphase spreads, was bimodally distributed with peaks centering on 92 and 93 (2n) chromosomes per cell.

The electrophoretic mobility of enzymes obtained from A-72 cells was identical to that of enzymes expressed by canine peritoneum cells and canine kidney cells. Thus, expression by A-72 cells of the 6 enzymes examined was typically canine.

Of the 7 canine viruses tested, 4 produced readily recognizable CPE in cultures of A-72 cells and a 5th virus (canine parainfluenza SV5) was detectable by hemadsorption (Table 1). The susceptibility of A-72 cells to each of these 5 viruses was essentially equivalent to that observed with the appropriate reference cell type. A-72 cells were not susceptible to infection with either canine distemper virus or minute virus of canines.

Development of a canine cell line susceptible to infection with several canine viruses provided a significant diagnostic and research advancement. The majority of cell lines available for virus diagnostic and research work are of human or rodent origin. Relatively few cell lines of canine origin have been established (11,17,18,19). In view of the host specificity of many viruses, a greater variety of canine cell lines are a necessity for continued expansion of studies of canine viruses and the diseases they cause. Reliance upon primary canine cell lines is often cost prohibitive, as well as time consuming. Therefore, the susceptibility of A-72

cells to infection with 5 of the 7 canine viruses tested indicated the definite potential of A-72 cells in the study and isolation of these viruses, and possibly others.

Morphologic characteristics, growth parameters, proliferative capacity, and media requirements of A-72 cells have not changed significantly throughout 135 passages. Thus, the criteria have been fulfilled for an established cell line. The chromosome composition of A-72 cells ($2n = 92$ to 93), however, was an unexpected finding, as canine cells normally contain 78 chromosomes. Definition of which chromosomes were present in increased numbers will be known upon completion of the karyotype analysis. Expression of only canine functions by A-72 cells was substantiated by the enzyme analyses.

A cell line (A-72) for canine virus studies was established from a tumor surgically removed from a female, 8-year-old Golden Retriever dog. Following explant culture, the cells have been serially passaged 135 times. A-72 cells have maintained a fibroblastic appearance and have a population doubling time of approximately 19 hours. Karyotypic analysis showed the modal $2n$ chromosome number to be 92 to 93. Using starch gel electrophoresis for enzyme characterization, the electrophoretic mobilities of enzymes extracted from A-72 cells were identical to those of canine peritoneal fibroblasts and primary canine kidney cells. The A-72 cells were susceptible to infection with infectious canine hepatitis virus, canine adenovirus type II, canine herpesvirus, canine parainfluenza virus, and canine coronavirus, but were not susceptible to canine distemper virus or minute virus of canines. These cells have been particularly useful for studies of the fastidious canine coronaviruses, as the commonly used primary canine kidney cells exhibit varied susceptibility to these viruses.

4. Respiratory disease in recruit military dogs: Canine parainfluenza virus infections in vaccinated dogs.

Numerous epizootics of respiratory disease have occurred from 1965 to 1974 in US military dogs at the recruitment and training centers. During the outbreaks, approximately 20% of the dogs developed signs of disease with many dogs being affected for 10 to 14 days. Etiologic studies incriminated canine parainfluenza (CPI) virus as the causative agent in each outbreak, and serologic tests showed that almost all of the susceptible dogs became infected (20,21).

A multivalent vaccine containing attenuated CPI virus was

licensed by the USDA in December 1974. Following experimental challenge, dogs given the CPI virus-containing vaccine did not develop signs of disease, but were infected as evidenced by excretion of virus and rises in antibody titers (22). The manufacturer recommended that the vaccine be given by either the subcutaneous (SC) or intramuscular (IM) routes, although the latter route was slightly more antigenic. Because the evaluation data on the vaccine was limited, a field trial in military dogs was initiated before adoption of the vaccine for routine use. The results of this study indicated that the vaccine did not produce any untoward reactions, the CPI virus did not spread to contact dogs, and significant levels of serum neutralizing antibodies developed in almost all dogs receiving 2 IM injections (2). Therefore, a recommendation was made in November 1977 to use the new vaccine for routine vaccination of US military dogs, with the specific recommendation to administer 2 IM doses of vaccine 30 days apart followed by an annual booster dose thereafter. Continued surveillance of respiratory disease among military dogs also was suggested due to the limited data on efficacy of the product under prolonged field usage. The purpose of this report is to summarize the laboratory observations on a small outbreak of respiratory disease that occurred in December 1977 among military dogs at the Department of Defense (DOD) Dog Center, Lackland Air Force Base, TX.

A limited epizootic of respiratory disease occurred at the DOD Dog Center between 6 and 9 December 1977. During the 4-day period, 33 of 320 (10%) unvaccinated dogs and 3 of 20 (15%) vaccinated dogs developed signs of upper respiratory infection. Two of the vaccinated dogs had received 2 SC injections of CPI vaccine and the third vaccinated dog had been given 1 IM injection. The severity and duration of clinical illness among the 3 vaccinated dogs essentially was identical to the syndrome exhibited by the unvaccinated dogs.

Acute and convalescent serum samples were obtained from each of the clinically ill dogs. Increased CPI virus neutralizing antibody titers were demonstrated in 27 of the 33 (82%) affected dogs. Of the 3 vaccinated dogs, 2 had significant rises in antibody titers, while the third dog had high levels of neutralizing antibodies in both serum samples. These observations clearly indicated that CPI virus infections were associated with the disease outbreak. Although each of the vaccinated dogs that became ill had not been immunized in the recommended manner, the findings demonstrated the necessity for continued surveillance of respiratory disease and CPI vaccine evaluation among military dogs.

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 012 Diseases of the Military Dog

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Table 1. Canine Viral Spectrum of A-72 Cell Line

Virus (strain)	Viral Seed Suspension		Titer ^a of Virus in A-72 Cells	
	Reference Cell	Titer ^a	Passage 18	Passage 129
Infectious Canine Hepatitis Virus (Cornell)	PDK ^b	5.5	5.0	6.0
Canine Adenovirus Type II (C9552)	PDK	3.5	2.5	2.5
Canine Herpesvirus	PDK	4.5	3.0	3.5
Canine Parainfluenza SV5 (3 X 84)	PDK	6.5 ^c	3.5 ^c	5.6 ^c
Minute Virus of Canines (2 X 66R)	WRCC ^d	2.0	< 1.0	< 1.0
Canine Coronavirus (1-71) (17B8)	PDK PDK	4.5 4.0	3.0 3.5	5.5 4.5
Canine Distemper Virus (Onderstepoort)	Vero	4.5	< 1.0	< 1.0

^aTiters expressed as -log 10/0.1/ml. ^bPrimary dog kidney cells. ^cTiter determined by hemadsorption of guinea pig red blood cells. ^dWalter Reed canine cell line.

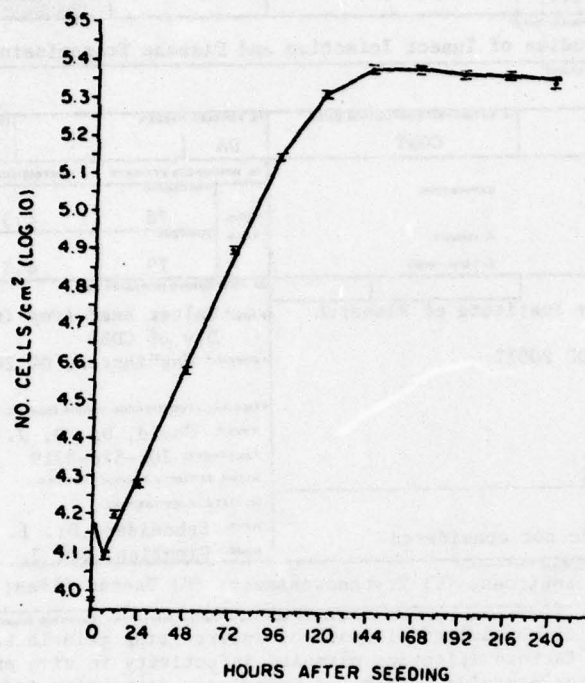


Fig. 1. Growth curve for A-72 cells in monolayer culture. The magnitude of the standard errors of the means is indicated by the horizontal bars.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
				DA OA 6514	78 10 01	DD-DR&E(AR)434	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGARDING ^a	8A. DISSEM INSTR ^a	8B. SPECIFIC DATA - CONTRACTOR ACCESS ^a	9. LEVEL OF DISSEM ^a
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10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62770A	3M162770A802		00		013	
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11. TITLE (Precede with Security Classification Code) ^a							
(U) Biological Studies of Insect Infection and Disease Transmission							
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002600 Biology							
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17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE: NA				PREVIOUS		b. FUNDS (in thousands)	
b. NUMBER: NA				FISCAL YEAR		260	
c. TYPE: NA				CURRENT		327	
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20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Div of CD&I			
				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursue DDAR if U.S. Academic institution)			
NAME: Rapmund, COL G.				NAME: Gould, Dr. D. J.			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3719			
22. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME: Schneider, Dr. I.			
				NAME: Gingrich, CPT J.			
23. REVISIONS (Precede EACH with Security Classification Code)							
(U) Malaria; (U) Mosquitoes; (U) Trypanosomiasis; (U) Tsetse flies; (U) Immunization							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Pursue individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) Development of physiological means of interrupting malaria transmission through an understanding of factors affecting parasite infectivity in vivo and in vitro. Develop test systems for studying mechanisms underlying sporozoite induced immunity for the eventual prevention and control of malaria in military troops. Develop model for the transmission of African trypanosomiasis in the laboratory and for utilizing the parasites in studies involving the immune response.</p> <p>24. (U) Determine quantitatively such parameters as the minimum numbers of immune cells required to convey protection against Plasmodium berghei malaria. Isolation of different stages of the malaria parasite on density gradients for subsequent study in culture systems. Establish a self sustaining colony of tsetse flies followed by the cyclic transmission of trypanosomes between insect vector and rodent host. Screen various vertebrate and invertebrate cell cultures for the propagation and differentiation of the blood stages and insect cycle, respectively, of Trypanosoma rhodesiense.</p> <p>25. (U) 77 10 - 78 09 Protection against Plasmodium berghei sporozoites could be conferred on recipient animals with sensitized spleen cells provided the recipients were semi-lethally irradiated prior to cell transfer and boosted 24 hours after transfer with 10,000 irradiated sporozoites. Without this augmentation, simple adoptive transfer of resistance with immune cells is not possible. Trypanosoma rhodesiense has been transmitted to tsetse flies but the percentage of mature infections found in the salivary glands is very low. Two continuous vertebrate cell lines are now used to support the growth of infectious bloodstream forms of Trypanosoma rhodesiense. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.</p>							

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1370

Project 3M762770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 013 Biological studies of insect infection and disease transmission

Investigators

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PFC John F. Shaker

Description

The major objectives of this work unit have been (1) the establishment of a self-sustaining colony of tsetse flies, (2) the development of a rodent/fly/rodent transmission model for Trypanosoma rhodesiense and (3) screening various primary and continuous cell cultures from both vertebrate and invertebrate species for the propagation and differentiation of the blood and insect stages, respectively, of a number of African trypanosomes. In addition, collaborative studies with personnel in the Department of Immunology, WRAIR, have focused on the effects of exposure to lymphocytes on the infectivity of Plasmodium berghei sporozoites (with CPT James K. Lovelace) and the use of liposomes to treat experimental malaria (with LTC Carl R. Alving). Progress on the latter two projects is covered in the annual report of the Department of Immunology.

Progress

1. Colony Establishment of the Tsetse Fly, Glossina morsitans

Significant progress has been made towards achieving a totally self-sufficient colony of tsetse flies for use in experimentation with T. rhodesiense infections. At this point in 1977, our laboratory-reared female population stood at ≈ 100 , whereas today the female population is greater than 1,200. This improvement can be largely attributed to changes in the flies' blood source, New Zealand rabbits, and more specifically to improved rabbit diets. A severe population decrease was experienced in January 1978, which was apparently due to an unknown toxicant in the rabbit diet, NIH 09. Switching to NIH 34m (guinea pig chow) and autoclaving it prior to feeding resulted in an immediate curtailment of mortality problems and increased fecundity (Table 1). However, autoclaving the diet was thought to cause vitamin deficiencies in the rabbits, so a vitamin fortified, autoclavable diet, NIH 32, was

obtained which alleviated this difficulty without any negative impact on fly survival or fecundity (Table 1). The primary indicators of colony vigor are mean puparial weight, mean adult mortality, average puparia per female, and percent adult eclosion. Of these four, three can be expressed by the term, the innate capacity for increase or Γ_m . This value can be derived as follows:

$$\Gamma_m = \log_e \frac{(N_t/N_0)}{T}$$

where N_t = Number of individuals at time t

N_0 = Number of individuals at time 0

T = interval over which population is observed

The Γ_m obtained using both the guinea pig diet and the NIH 32 diet was 0.0124, a 35% improvement over the old rabbit diet, NIH 09 ($\Gamma_m = 0.0080$).

Although theoretically an optimal female population size of ≈ 2000 can be achieved by rearing flies on rabbits, this procedure is labor-intensive (5-6 man hours/day) and expensive in terms of rabbit per diem costs (a minimum of 32 rabbits must be maintained). It is therefore desirable to convert the colony to an in vitro feeding system as has been done at most other large-scale rearing activities. This would require approximately 750-ml of pig blood per week. Up until early July of this year the donor sow at the WRAIR Farm was maintained on a standard hog diet which contained an abundance of antibiotics. In early July the sow was placed on an antibiotic-free diet. The results presented in Table 2 reflect the effects of antibiotic both on average daily reproduction and on mean puparial weight. Both of these were considerably improved when the sow was shifted to an antibiotic-free diet (Table 2). However, the average daily reproduction of in vitro-fed flies still lagged far behind that of flies maintained on rabbit blood alone; each female fed on pig blood was replacing herself 1.6 times over the course of her 90 days of reproductive life while each rabbit-fed female was replacing herself 2.5 times over the same period.

2. Development of a Cyclical Transmission Model

A strain of T. rhodesiense (LVH), obtained from a Kenyan in 1975 and subsequently passed in rodents, was used to obtain stabilates for immunological studies on antigenic variation as well as for studies on cyclical development in tsetse flies.

Flies less than 48 hours old were fed either directly on C57BL/6 mice infected with trypanosomes from the first parasitemic

peak or else they obtained an infective meal of the same trypanosome isolate purified through a cellulose column, resuspended in horse, pig, or rabbit blood, and finally administered through an in vitro feeding system to enhance uniformity of infection. Following the infective meal, the flies were maintained on a rabbit for 18-21 days, then switched to mice to determine which flies had actually obtained a mature salivary gland infection. This was done by feeding single flies on single mice and checking daily by using wet blood mounts from mouse tails. Between 3-40 days post-infection, dead or moribund flies were removed daily and prepared for histological examination of the midgut, hindgut, hemocoel, proventriculus, and salivary glands. The presence of trypanosomes was recorded as either heavy, moderate, or light depending on the relative numbers found in each tissue.

The complete regimen of tests conducted is shown in Table 3. The usual isolate of choice for infection was LVH. However in test 10 a strain (MFM) previously passed through a fly was used to determine if cyclical passage caused enhanced infection rates. The general thrust of these tests was to determine conditions for enhanced infection rates in flies. Simultaneously, a predictive model was to be developed that would allow one to predict salivary gland infection rates by examining infection rates in midguts.

The data in Table 3 suggest that because overall infection rates are very low any differences are likely to be subtle. A total of 12 flies achieved mature infections in 8 experiments; two experiments failed to yield infected flies. Of the 12 infected flies, one became infective 18 days post-infection (Test 10, the fly-passed isolate), and in test 9 the latest infection was obtained from a fly 29 days post-infection. All the other flies became infective between 22 and 28 days post-infection. In most instances a normal mouse would develop a parasitemia 72 hours after an infected fly bite.

The sequence of development of an infection in flies is from posterior midgut (PMG) to anterior midgut (AMG) to proventriculus (PV) to salivary glands (SG), and each of these stations represents a barrier to further cyclical development; thus, the relationship between the PMG and AMG infection rates and the SG infection rate are quite important. Theoretically each station in the development of infection should display a lower rate than the preceding station and a higher rate than the succeeding station. In general terms this is true. However, the lack of a difference between PMG and AMG infection rates is most likely due to the fact that relatively few flies were collected less than 7 days post eclosion, meaning that many PMG infections must have died out early while those that persisted into AMG infections usually also retained

a PMG infection. As was expected, PV infection rates ranged from 0 to 100% (average = 45%) of the AMG rates while SG rates ranged from 0 to 60% of the AMG rates (average = 33%) and 49 to 100% of the PV rates (average = 78%). The two biggest barriers would hence be the low initial PMG infection rate and the low proventricular infection rate. To date, there have been no cases where a proventricular infection rate was obtained and a salivary gland rate was not.

The data presented by Buxton (1) (1955, after Burt 1946) showed mature infection rates of *T. rhodesiense* in *G. morsitans* ranging from 1.6 to 5% in the field and from 7.6 to 14.7% in the laboratory, depending on trypanosomal isolate. Our results showed no significant difference between flies infected on parasitemic mice (tests 7 and 9) and those fed through a membrane on parasites resuspended in horse blood. The highest mature infection rates with the LVH isolate occurred with 5 and 6-day parasitemic mice (average = 3.2%) while the MFM isolate had a significantly higher rate of 5.6%. Jenni (4) (77) using *T. brucei*, found mature infection rates of up to 44% using special infection procedures which we are attempting to duplicate. However, even the most judicious application of Jenni's procedures is not expected to yield nearly such high rates considering the species and general low infectivity of this particular isolate. Still more work needs to be done in not only improving infection rates of isolates, but also in achieving clonal infections in flies.

3. Immunization/Challenge Tests with Infected Tsetse Flies

An infected fly containing trypanosome isolate MFM 369 B6 was dissected and tissues separated to yield salivary glands (SG), midgut (MG) and proventriculus (PV). The tissues were then separately macerated in a syringe with fetal calf serum. Two mice were injected with the irradiated suspension obtained from each macerate. Mice were estimated to have received the following numbers of trypanosomes.

Mice 11-SG 1 & 2 = 10^6 trypanosomes each

Mice 11-MG-3 & 4 = 6×10^5 trypanosomes each

Mice 11-PV-5 & 6 = 3.8×10^4 trypanosomes each

Five other mice were simply injected with 0.5 ml each of fetal calf serum and were designated 11-FCS-1,2,3,4, & 5. Table 4 summarizes the results of a challenge of these mice approximately 3 months later by the bite of a fly infected with LVH trypanosomes. A first infection was always obtained on day 4 or earlier, suggesting no protection from the immunization procedure. These mice

all received Berenil treatment sometime during or shortly after the 1st parasitemic peak. These same mice were then challenged with the same fly (2nd infection) 2 weeks or more after Berenil treatment. Some protection was afforded all mice which were cured by Berenil treatment after the 1st parasitemic peak with delays in parasitemia ranging from 5 days in 11-MG-3 to 19 days in 11-SG-2. Similarly these mice were again challenged (3rd infection) with a different fly infected with LVH in test 13. Delays in parasitemia were again experienced with 11-FCS-1 and 11-SG-2 experiencing no parasitemia up to day 25 and 30 post-infection, respectively.

A repeat of this experiment was then attempted using the tissues from the infected fly in test 11 for immunization. The immunizations and mouse numbers were assigned as follows:

<u>Mouse #</u>	<u>#Tryps injected</u>
13-SG-1	3.5×10^5
13-SG-2	3.5×10^5
13-MG-1	4.3×10^7
13-MG-2	4.3×10^7
13-PV-1	10^6
13-PV-1	10^6
13-FCS-1,2,3,4	none

These mice were then challenged with the infected fly from test 13 with the results shown in Table 5. The fetal calf serum (FCS) controls had been injected with Berenil and allowed to clear for 2 weeks before a first infection attempt. The first challenge on the immunized mice occurred within 1 month after vaccination with trypanosomes to avoid possible loss of immunity. No protection was afforded by immunization with fly-form trypanosomes, at least in the numbers used in these tests (Table 5). The reason for lack of parasitemia in one control mouse (13-FCS-1) is not clear, but may indicate that the fly had temporarily lost infectivity. The curing of a prior parasitemia appears to afford some protection against the same isolate (passed through 2 different flies) and suggests that some form of immunization may yet be possible.

4. Cultivation of Trypanosoma brucei brucei and T.b. rhodesiense in Vertebrate and Invertebrate Cell Lines

In 1977, Hirumi et al., (2,3) reported the successful cultivation of T.b. brucei (strain 427) using a bovine fibroblast-like cell line and RPMI 1640 medium supplemented with 20% fetal bovine serum. The trypanosomes so grown retained the morphological characteristics of the long, slender bloodstream trypomastigotes, including the surface coat, and were infective for mammalian hosts, although with ever decreasing virulence. The potential of such a system for obtaining large numbers of parasites for vaccine purposes, for biochemical studies and for insight into the mechanisms of antigenic variation is readily apparent and the purpose of the current research was to test the possibility of using the same ILRAD cell line or other cell lines to grow the bloodstream forms of a number of African trypanosomes.

Four isolates of T. brucei were used: canine 73 and strain 427 of T.b. brucei and the Wellcome and LVH isolates of T.b. rhodesiense. The cell lines, listed in Table 6, were obtained from other departments at WRAIR, from the American Type Culture Collection, Rockville, MD, or, in the case of the ILRAD line, from Dr. H. Hirumi, Nairobi, Kenya. Prior to receiving the ILRAD line, preliminary work indicated that the L929 cell line showed the most promise in supporting the growth of the trypanosomes and hence, it was used most often for comparative studies once the ILRAD line was available.

Early experiments with the canine isolate of T.b. brucei involved the above two cell lines. Initially, a total of 10 flasks of L929 cells and 8 of ILRAD cells were inoculated with $0.8 - 7$ and $2 - 6.4 \times 10^4$ trypanosomes/ml, respectively. The individual flasks were subcultured when the count of trypanosomes per ml exceeded by 50% the count of the initial inoculum. Following subculturing, the trypanosomes were again counted in the old as well as in the new flask. Periodically, an aliquot (usually 0.5 ml) of the parasite suspension was removed from the flasks and inoculated into normal mice to test for infectivity. The parasites in the ILRAD cells survived a maximum of 7 days. Only on the second day did 2 of the flasks require subculturing. In the remaining flasks the parasites did not increase in number but remained either approximately equal to the initial number or began to die very quickly. The parasites in all of the flasks began to decrease by day 3 or 4 and all of the flasks were discarded by day 7. In contrast, the parasites cultured with the L929 cells initially grew with great rapidity. It was not uncommon to subculture many of the flasks two times a day due to a 3-5 fold increase in the number of parasites. Efforts to moderate the growth to levels approaching $1-5 \times 10^5$ /ml, by additional subculturing or by

withdrawing and renewing medium, during a 24 hour interval were unsuccessful. Failure to stabilize the growth rate led to overcrowding and the subsequent formation of bizarre forms followed by declining numbers of parasites 5-7 days after the initial subcultivation. Trypanosome populations maintained in vitro for 1-2 weeks were infective, the mice showing positive blood films by day 4 or 5. Older populations were only rarely infectious.

Hirumi et al., in their second paper (3) indicated that relatively low numbers of parasites in the initial inoculum usually resulted in a high success rate in subsequent subcultures. Thus, a second series of experiments involved placing four different densities of T.b. brucei in L929 and ILRAD cell cultures. Four replicates at each density were used for each of three experiments. The range of densities was as follows:

Very low:	$0.51-1.30 \times 10^2$
Low:	$1.28-2.56 \times 10^3$
Medium:	$1.28-1.60 \times 10^4$
High:	$1.40-2.20 \times 10^5$

When required, the parasites were subcultured to maintain a population density of $1-5 \times 10^5$ trypts/ml in any of the flasks. Periodically, aliquots of the suspensions were injected into normal ICR mice.

Tables 7 and 8 show the results at various densities in ILRAD and L929 cells, respectively. All of the parasites in the ILRAD cultures died by day 7. The only group that did reasonably well for the first 3 days was that started with a very high density of trypanosomes (1.52×10^5 /ml). These results are in complete contrast to those described by Hirumi et al., (2) in that they were able to successfully subcultivate and maintain trypanosomes originating from cultures started with densities as low as 1.6×10^2 trypts/ml.

The results of using L929 cells (Table 8) were somewhat more encouraging. Again the only group which showed promise was that started with a high density (1.58×10^5 trypts/ml). Trypanosomes started in cultures at low densities died by the 7th day. Table 9 shows the progressive subcultivation of the canine strain of T.b. brucei from a single flask of L929 cells for the first 10 days. During this period 14 additional flasks of cells were required for subculturing the parasites. Once again, however, it was not possible to stabilize the growth rate with the exception of one group of flasks from a subculture on day 5. In this series, reducing the trypanosome number to approximately 1.5×10^5 /ml on any one day would lead to a population of

4 - 6×10^5 /ml 24 hours later. This growth pattern was successfully maintained for three weeks with the parasites retaining infectivity for mice. By contrast, trypanosomes cultured with the ILRAD cells for as little as 24 hrs were no longer infectious.

A fairly wide range of vertebrate cell lines were screened for their ability to support the growth of the Wellcome isolate of T.b. rhodesiense (see Table 6). The L929 cell line served as a control. Initial populations ranged from $0.2 - 1.38 \times 10^5$ tryps/ml and the flasks were subcultured when the parasite density exceeded 2×10^5 /ml. Another series of experiments involved placing 4 different densities of the Wellcome isolate of T.b. rhodesiense into L929 and ILRAD cells. The range of the different densities in tryps/ml was:

- Group 1: $1.4 - 1.6 \times 10^4$
- Group 2: $2.0 - 5.8 \times 10^4$
- Group 3: $1.9 - 7.0 \times 10^5$
- Group 4: $1.78 - 2.35 \times 10^6$

The flasks were subcultured when the densities exceeded by 50% the initial number of trypanosomes. Usually every 48 hours an aliquot was removed from each flask and inoculated into normal ICR mice.

The L929 cells supported growth of the Wellcome isolate better than any other cell line. The average life span of the parasites in L929 cells was 11 days although in one experiment they survived for 26 days. The average life span in the other cell lines was as follows:

Bov. Emb. Kid.	4 days
Canine peritoneum	4 "
Primary green monkey kidney	5 "
Hela 199/10%FBS	8 "
Hela 199/20%FBS	6 "
Hela 1640/20%FBS	5 "
Hela 199/20%HS	6 "
ILRAD	5 "

The results of placing different densities of Wellcome tryps in L929 cells seemed to indicate that low densities were better than high densities. Generally cultures started with densities

of less than 5.8×10^4 trypts/ml survived for approximately 14 days and throughout this interval were infective for mice. Cultures initiated with densities greater than 1.9×10^5 trypts/ml survived for only 8 days.

The results of placing different densities of the Wellcome isolate in ILRAD cells were invariably negative. None of the densities survived for more than 5 days and there did not appear to be any differences between the various density groups. Furthermore the great majority of mouse inoculations were negative.

An initial experiment comparing the growth of the LVH isolate in 4 different vertebrate cell lines (L929, Bov. Emb. Kid., Hela and ILRAD) was equally unpromising. The initial number of trypanosomes ranged from $6 - 2 \times 10^4$ trypts/ml. In none of the flasks did the parasites survive for more than 4 days. Since this particular isolate is a fairly recent one and still infective to tsetse flies, LVH as well as the Wellcome isolate were inoculated into cultures containing Glossina morsitans cells. The starting densities for the LVH and Wellcome parasites were $4 - 9 \times 10^4$ /ml and $2 - 3 \times 10^5$ /ml, respectively. Figure 1 shows the striking difference in the response of the trypanosomes to the tsetse fly cells. Whereas the Wellcome isolate survived a maximum of 8 days without any evidence of multiplication, the LVH trypanosomes multiplied with ever increasing rapidly and apparently can do so indefinitely as long as the cell layer remains in healthy condition. The infectivity of the trypanosomes is variable and has not, as yet, been ascribed to any one factor or set of factors. What is clear is that the cells per se make some contribution as the parasites often grow as well in medium alone as in medium with cells, but infectivity is lost by day 4 or 5 in the former and not until day 7 - 16 in the latter.

The inability to repeat Hirumi's results with the aforementioned strains of T. brucei prompted a request to Dr. B.M. Honigberg, University of Massachusetts, for the 427 strain of T.b. brucei. These parasites were cultured in 4 different cell lines; namely, CCL 16, CCL 39, CCL 40 and the ILRAD line (see Table 6). The first 3 lines were suggested by Dr. Hirumi as "probably adequate" substrates for the parasites. Cultures were inoculated with the concentrations varying from $0.5 - 2.6 \times 10^5$ /ml. Thereafter, a population density of about 10^6 /ml was maintained. Unlike most of the other strains worked with, trypanosomes from the 427 strain usually decreased in number following inoculation into any of the 4 cell lines and did not show many dividing forms until day 3 or 4. Thereafter, by replacing the medium by one-quarter or one-third on a daily basis, the growth rate of trypanosomes could be stabilized fairly well (see Table 10 for a typical

series). Cultures in which the parasite counts exceeded $5 \times 10^6/\text{ml}$ in a 24 hour interval were usually impossible to recover. Most often, the parasites appeared incapable of complete division, with masses of up to 10 or 12 trypanosomes fused along $1/3$ to $1/2$ their length. Others appeared much thicker and moved much more slowly than normal. Although the trypanosomes grew well in all of the lines, the CCL 16 line has a slower growth rate and for that reason is preferred over the others, i.e., there is less subculturing involved in maintaining the trypanosomes for extended periods of time. Thus far, the trypanosomes have been cultured up to 35 days without loss of infectivity.

Preliminary attempts to grow the LVH isolate in the CCL 16 cell line have been unsuccessful, the parasites not surviving more than 4 days. It thus appears that the bloodstream forms of the different isolates of T. brucei have very different and selective requirements for growth outside of the vertebrate host. The other cell lines have yet to be tested.

Conclusions and recommendations

1. Although a self-sustaining colony of approximately 1,200 female tsetse flies is now operational, a totally self-sufficient colony of 2,000 females is required to produce some 250 surplus puparia per week for experimental purposes. This goal is obtainable using rabbit-fed flies, but labor and cost saving considerations dictate that the colony be switched over to in vitro feeding on defibrinated pig blood as soon as possible. This will be effected as soon as data on the in vitro fed flies indicates that no adverse effects on colony vigor will occur.

2. Cyclical transmission of T. rhodesiense through flies is occurring at a predictable, though low, infection rate. Further efforts to improve infection rates by manipulation of environmental conditions can be expected to yield positive results based on experimental work to date. This work is being hampered by lack of space in which to place the needed equipment. A larger tsetse-fly laboratory would greatly enhance the probability of success. In addition to work on cyclical passage of T. rhodesiense isolates, additional work on cyclical passage of clones needs to be pursued in the coming year.

3. Immunization and challenge experiments with fly-form trypanosomes have not yet produced an animal immune from fly bite. However, experiments with Berenil-cured mice have resulted in enhanced, if incomplete, immunity, especially if combined with a prior immunization with fly-form trypanosomes. These studies need to be pursued further, primarily by obtaining larger numbers of infected flies for immunization and challenge.

4. The method developed by Hirumi and his colleagues for culturing infectious bloodstream forms of African trypanosomes may not apply to more than a limited number of isolates of either T.b. brucei or T.b. rhodesiense. Nonetheless, the focus should be on the strain 427 - ILRAD model system until it is thoroughly understood and the cultures can be "read" far more accurately than is presently the case. Once that is accomplished, efforts can be directed toward culturing other isolates in other cell systems.

5. The fact that the LVH isolate can be maintained in a tsetse fly cell line and not in any of the vertebrate cell lines tested is quite intriguing and should be further explored. One obvious direction is to compare the culture media (composition, pH, osmolarity, etc.) and the utilization of medium components by the cultured cells with and without the trypanosomes. Other in-vertebrate cell lines should also be screened to determine whether or not vector-specific factors are involved.

TABLE 1
Comparison of Colony Vigor Measured in Female Flies Fed on New Zealand Rabbits
Maintained on Various Diets

Host Food	Fly Generation	Mean Number puparia per ♀	Mean weight (mg) ± S.E.	± Adult Eclosion	Mean longevity (days)	Innate Capacity for Increase r_m
NIR 09	Parental	4.8	29.6 ± 1.0	81	117.9	0.0080
NIR 09, NIR 34M (Autoclaved)	F ₁	6.3	30.4 ± 0.5	89	89.5	0.0124
NIR 34M, NIR 32 (Autoclaved)	F ₂	6.7	31.7 ± 0.5	85	93.8	0.0124
NIR 32 (Autoclaved)	F ₃	-	30.9 ± 0.4	89	-	-

TABLE 2

Effects of antibiotic-containing and antibiotic-free diets on female flies maintained in-vitro on pig blood as compared to flies maintained on New Zealand rabbits (NZR). Measures included average daily mortality (ADM), average daily reproductivity (ADR) and mean puparial weights (in mg \pm S.E. = standard error)

	Test 2 (Antibiotic diet)			Test 3 (Antibiotic-Free)			Test 4 (Antibiotic-Free)		
	ADM	ADR	Mean Pup. weight	ADM	ADR	Mean pup. weight	ADM	ADR	Mean pup. weight
NZR	0.96	4.06	28.7 \pm 0.5	0.97	3.63	28.0 \pm 0.7	0.89	5.5	29.5 \pm 1.0
In Vitro	1.20	1.02	26.5 \pm 1.1	0.87	2.86	28.5 \pm 0.7	1.46	3.5	27.8 \pm 0.7

TABLE 3

Posterior midgut (PMC), anterior midgut (AMG), proventricular (PV) and salivary gland (SG) infection rates in flies fed on infected mice of varying parasitemia and in flies fed on different isolates (LVH and MVM) and different blood sources (tests 11-13) through a membrane. Actual numbers are given of histologically examined flies (PMC, AMG and PV) and mouse probing tests (SG). Numbers in parentheses indicate percentages.

Test #	Tryp Strain	Infection Method	Parasites	Pre/Post Treatment Condition	PMC Inf (%)	AMG > 7 days Inf (6%)	PV Inf > 10 days (%)	SG Inf > 16 days (%)
6	LVH	5 day membrane (horse)	10^7	25/21	0/22(0)	0/22(0)	0/22(0)	0/20(0)
7A	LVH	4-day mouse	0	-	-	-	-	-
7B	LVH	5-day mouse	10^6 - 10^8	25/21	0/6(0)	0/5(0)	0/6(0)	0/10(0)
7C	LVH	6-day mouse	10^7 - 10^8	25/21	3/20(15.0)	1/20(5.0)	1/20(5.0)	1/18(6.0)
7B&C					3/26(11.5)	3/25(12.0)	1/26(3.8)	1/28(3.6)
8	LVH	5-day membrane (horse)	10^7	25/20	2/54(3.7)	2/54(3.7)	1/52(1.9)	1/61(1.6)
9A	LVH	4-day mouse	10^6	25/20	1/26(3.8)	1/26(3.8)	0/26(0)	0/25(0)
9B	LVH	5-day mouse	10^6 - 10^7	25/20	2/27(7.4)	2/26(7.7)	2/26(7.7)	1/26(3.8)
9C	LVH	6-day mouse	10^7 - 10^8	25/20	2/27(3.7)	1/27(3.7)	0/25(0)	1/13(0)
9A,B,C					4/80(5.0)	4/79(5.1)	2/73(2.7)	1/64(1.6)
9B&C					3/54(5.6)	3/53(5.7)	2/51(3.9)	1/39(2.6)

TABLE 3 (Continued)

Test #	Tryp Strain	Infection Method	Parasites	Pre/Post Treatment Condition	PMG Inf (2)	AMC > 7 days Inf (6 2)	PV Inf > 10 days (6 2)	SC Inf > 16 days (6 2)
10	MPH	5-day membrane	10 ⁷	25/20	3/32 (9.4)	3/32 (9.4)	2/32 (6.3)	2/36 (5.6)
11	LVI	5-day membrane (horse/saline)	10 ⁷	25/20				1/48 (2.1)
12	LVI	5-day membrane (1.0% glucose)	10 ⁷					0/115 (0)
		Pig cells/saline (0.1% glucose)						
13	LVI	5-day membrane (rabbit or horse)	10 ⁷	28/20				3/101 (3.0)

TABLE 4

Challenge of mice immunized with fly-form trypanosomes (SG, MG & PV) and non-immunized (FCS & control) mice by fly bite of test 11 fly (LYN) before Berenil treatment (1st infection), after 1st Berenil treatment (2nd infection) and by fly bite of test 13 fly after a 2nd parasitemia and 2nd Berenil treatment.

Mouse Number	1st Infection	1st Parasitemia	1st Berenil Treatment	2nd Infection	2nd Parasitemia	2nd Berenil Treatment	3rd Infection	3rd Parasitemia
11-SG-1	10 June	13 June	22 June	3 Aug	15 Aug	15 Sept	-	-
11-SG-2	16 June	19 June	22 June	14 July	1 Aug	19 Aug	1 Sept	Clear up to 2 Oct
11-MG-3	19 June	22 June	22 June	25 July	2 Aug	2 Sept	18 Sept	Clear up to 2 Oct
11-MG-4	21 June	23 June	Died in treatment	-	-	-	-	-
11-PV-5	22 June	26 June	Died in treatment	-	-	-	-	-
11-PV-6	25 June	28 June	30 June	30 July	12 Aug	29 Aug	22 Sept	Clear up to 2 Oct
11-FCS-1	none	none	22 June	22 July	25 July	23 Aug	4 Sept	30 Sept
11-FCS-5	none	none	22 June	10 July	13 July	11 Aug	29 Aug	7 Sept
11-436C2 Control (#4)	12 June	15 June	22 June	18 July	31 July	17 Aug	Died prior to infection	-

TABLE 5

Challenge of non-immunized or immunized mice with fly form trypanosomes (SG, MG, & PV) by bite of test 13 fly (LVH)

Mouse #	1st Infection	1st Parasitemia
13-SG-1	6 Sept	10 Sept
13-SG-2	12 Sept	16 Sept
13-MG-1	10 Sept	13 Sept
13-MG-2	-	-
13-PV-1	28 Sept	1 Oct
13-PV-2	-	-
13-FCS-1	8 Sept	none up to 2 Oct
13-FCS-2	16 Sept	19 Sept
13-FCS-3	24 Sept	27 Sept

TABLE 6

Source of cells tested as substrate for the cultivation of bloodstream forms of
I. b. brucei and I. b. rhodesiense

Designation of cell line	<u>I. b. brucei</u> isolates		<u>I. b. rhodesiense</u> isolates	
	canine 73	strain 427	Wellcome	LVN 18
L929 (mouse connective tissue)	X		X	X
Rovine embryonic kidney			X	X
Canine peritoneum			X	
HeLa			X	X
Primary Green Monkey kidney			X	
IL2AD 277	X	X	X	X
Glossina morsitans (WR3-Gm-1)			X	X
CCL 16 (Chinese hamster lung)		X		X
CCL 39 (Chinese hamster lung)		X		
CCL 40 (Buffalo lung)		X		

TABLE 7

Counts of bloodstream *T. b. brucei* in ILRAD cells initiated with various densities (at 34°C)

Initial Day Density	No. of Trypanosomes ($\times 10^5/\text{ml}$)						
	1	2	3	4	5	6	7
1.52×10^5	2.22 (1.30)	1.78 (1.22)	0.64	0.44	0.08 D		
1.60×10^4	NC	0.06	0	0	NC	NC	0 D
1.28×10^3	NC	NC	NC	0	NC	NC	0 D
1.30×10^2	NC	NC	NC	0	NC	NC	0 D

D - Discontinued

() - Number of trypanosomes after medium was changed

NC - Not counted, no medium changed

TABLE 8

Counts of bloodstream *T. b. brucei* in L929 cells initiated with various densities (at 34°C)

Initial Day density	No. of Trypanosomes ($\times 10^5/\text{ml}$)											
	1	2	3	4	5	6	7	8	9	10	11	12
1.58×10^5	19.20 (2.0)	12.80 (1.16)	1.40	3.48 (1.90)	4.86 (1.02)	1.00	2.08 (1.32)	1.42	1.44	0.98	0.30	0.14 D
1.6×10^4	NC	0.48	1.20	1.70 (0.70)	0.58	0.48	0.42	0.68	0.70	0.12 D		
1.28×10^3	NC	NC	NC	0.02	NC	NC	0 D					
1.30×10^2	NC	NC	NC	0	NC	NC	β					

D - Discontinued

() - Numbers of trypanosomes after medium was changed

NC - not counted, no medium changed

TABLE 9

Maintenance and subcultivation of bloodstream *I.b. brucei* in 1929 cells at (34°C)

Initial Density	No. of Trypanosomes ($\times 10^5$ /ml)									
	1	2	3	4	5	6	7	8	9	10
1.58	19.20 (2.00)	12.80 (1.16)	1.40	3.48 (1.90)	4.86 (1.02)	1.00	2.08 (1.32)	1.42	1.44	0.98
		1.70	2.26 (1.10)	0.76	1.46	0.46	0.10 D			
			0.70	0.44	0.48	0.16	0.14 D			
				1.00	1.66	1.34	1.32 (1.00)	3.14 (1.24)	0.62	0.08 D
					1.22	1.18	4.20 (1.46)	5.02 (1.78)	5.08 (1.12)	6.50 (1.76)
					1.04	1.18	3.50 (1.36)	3.82 (1.66)	4.12 (1.58)	6.50 (1.48)
							1.28 (1.72)	3.48 (1.72)	1.52	6.80 (1.42)
							1.58	4.40 (1.04)	1.50	7.44 (1.36)
								1.28	2.88 (1.26)	7.68 (1.22)
									1.30	11.20 (1.52)
									2.04	24.00 (1.86)
										8.40 (1.14)
										1.60 (1.14)
										1.98

() - No. of trypanosomes after
change of medium

D - Discarded

{ - Connects original and subcultured
flask(s)

TABLE 10

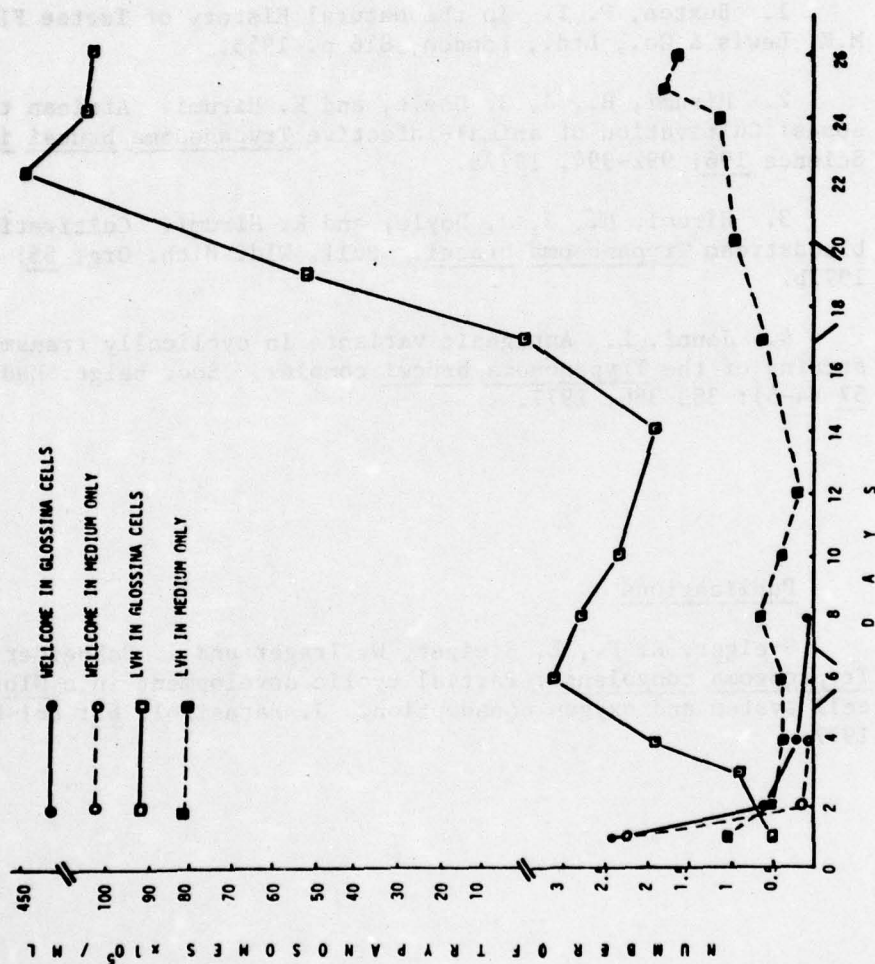
Growth of T. b. brucei (strain 427) in different vertebrate cell lines

Trypanosomes/ml ($\times 10^6$)				
Cell line Day	CCL 16	CCL 39	CCL 40	ILRAD
0	0.15	0.15	0.15	0.15
1	0.10	0.12	0.15	0.08
2	0.35*	0.15	0.60*	0.10
3	1.30	0.75*	1.70	0.30*
4	1.90	1.00	2.80	0.95
6	2.40	C	2.10	2.30
8	1.80	--	1.20	1.35

*Initial change of 1/4 to 1/3 of medium; thereafter at 24 hr. intervals

C - Contaminated

FIGURE 1.
COUNTS OF *BLUTISTREUM IMPROBANS* IN WELLCOME AND LVH ISOLATES IN GLOSSINA HOSTING CELLS



Project 3M162770A802 MILITARY PREVENTIVE

Work Unit 013 Biological studies of insect infection and disease transmission

Literature Cited

Reference:

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Steiger, R. F., E. Steiger, W. Trager and I. Schneider: Trypanosoma congolense: Partial cyclic development in a Glossina cell system and oxygen consumption. J. Parasitol. 63: 861-867, 1977.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OB 6538	78 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOWN INSTR	8B. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
77 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
A. PRIMARY	62770A	3M162770A802		00		014	
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NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
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TELEPHONE: 202-576-3551				TELEPHONE: 202-427-5109			
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				NAME: Eckels, Kenneth H., Dr.			
				NAME: Summers, Peter			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U)Arbovirus; (U)Dengue; (U)Vaccine; (U)Immunity; (U)Attenuation; (U)Tissue Culture							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) The objective is development, production, and assay of live-attenuated vaccines against classical strains of dengue viruses. The major types (1,2,3, and 4) of this virus are endemic throughout populated areas of the world, and although mortality rates are low, the incapacitation effected by these viruses and their associated sequelae could have serious impact on military time tables and troop mobility.</p> <p>24. (U) Selected strains are subjected to multiple passages and frequent cloning in tissue culture systems, to produce pure progeny characterized by reduced virulence and adequate antigenicity, that will serve as candidate vaccine seed virus.</p> <p>25. (U) 77 10 - 78 09 1. Following approval by in-house committees and by the Bureau of Biologics (FDA), lot 1 of a live-attenuated dengue-2 vaccine was tested in 2 groups of human volunteers totaling 6 individuals. Five recipients of the vaccine developed viremia, however there was no indication of reversion of the vaccine virus to a more virulent form. Two volunteers developed fever of short duration and one volunteer developed an erythematous rash. Five of the 6 volunteers seroconverted by various immunological tests. The one volunteer who did not have a viremia and who did not stimulate antibodies was found to have pre-vaccination antibodies to the vaccine virus and had a history of a previous infection with dengue-4 virus. 2. A human isolate of dengue-3 virus has been passaged and cloned in FRhL cells. One clone, labelled C-5, was shown to be temperature-sensitive in cell culture and was attenuated for mice and monkeys. Monkeys receiving the C-5 clone stimulated the production of antibodies and were protected against challenge with the parent virus. After a final series of 3 cloning-purification steps, a working (master) seed was prepared in roller flasks and tested for safety. The C-5 clone appears not to have changed phenotypically upon passage and will be used for the preparation of a production seed for dengue-3 vaccine. For technical report see WRAIR Annual Progress Report, 1 Oct 77 - 30 Sept 78.</p>							

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1395

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 014 Characteristics of attenuated dengue viruses

Investigators.

Principal: Venton R. Harrison, M.S.

Associates: Kenneth H. Eckels, Ph.D.; Peter L. Summers

I. Dengue-2 Vaccine Progress.

A. Safety and immunogenicity of DEN-2, PR-159, S-1 vaccine, lot #1 in adult volunteers. Six volunteers were divided into two groups and inoculated with a single dose (4.5×10^5 PFU, Group A and 2.5×10^5 PFU, Group B) of a live attenuated dengue type 2 vaccine according to protocol. All six volunteers were placed in protective isolation for 10 days post-vaccination. Two recipients developed fever (oral temp $> 100^\circ\text{F}$) after day 10 accompanied by leukopenia. One volunteer developed a fever to 101.8°F with headache, myalgia and photophobia which prevented him from returning to work. Another recipient developed an erythematous rash of the chest and abdomen which lasted from day 11 to day 17 post-vaccination.

Five recipients developed viremia 8 or 9 days post vaccination which lasted from 1 to 10 days. All 24 virus isolations from plasma produced the same small plaques in vitro as were produced by the vaccine virus. None of the isolates tested replicated at the nonpermissive temperature (39.3°C) which is restrictive for the vaccine virus. Thus, there was no evidence that infection of human volunteers led to a reversion to virulence as measured by reliable in vitro assays. Furthermore, in contrast to the findings in wild type dengue infections, virus was not isolated from the circulating monocytes of the vaccinees.

The immune responses were evaluated by hemagglutination inhibition (HI), complement fixation (CF) and plaque reduction neutralization tests (PRNT). All six vaccinees seroconverted by HI and five by CF and neutralization tests. Broadly reactive HI antibody in all five viremic volunteers were typical of secondary responses to a flavivirus infection. The serum antibody titer to dengue type 2 was found to be highest in the volunteers with the longest interval since their yellow fever immunization. The one volunteer without viremia was found to have pre-existing DEN-2 antibody to the vaccine parent virus but not to the prototype strain. Following immunization, he had no rise in neutralizing antibody and minimal rises in HI and CF antibodies.

At the dosage given, the DEN-2 vaccine infected 5 sero-negative recipients, but not the volunteer with pre-existing homologous neutralizing antibody. One of five susceptible volunteers developed mild dengue fever; all five volunteers developed typical secondary flavivirus immune responses. The vaccine is sufficiently safe and immunogenic to warrant future investigation of its dose response in human volunteers.

For details of the vaccine trial, see the Annual Report, 1978, Department of Virus Diseases, DCD&I.

B. Temperature-dependent reversion of S-1 vaccine virus.

The observation has been made that S-1 virus is less stable at temperatures approaching the non-permissive temperature of 39.0°C. Large plaque, revertant virus is found more frequently in cell cultures held at 35°C and 37°C than those held at 31°C (Annual Report, 1977). Multiplicity of infection (MOI) also effects the reversion phenomenon; cultures inoculated with a relatively high MOI are more likely to contain large plaque virus on harvest than those cultures inoculated with a lower MOI (1). In order to more fully substantiate the effect of temperature and MOI on the reversion of S-1 virus, 24 individual cell cultures were inoculated at two MOI's and incubated at temperatures of 31°C, 35°C, and 37°C. Table 1 lists the mean virus titers for 7 day harvests of each set of cultures. At 31°C, cell cultures inoculated with either a 0.7 or 0.007 MOI

Table 1. Effect of temperature and MOI on the reversion of S-1 virus.

Temperature	MOI	No. cultures with revertant virus/ total no. cultures	
31°C	0.7	0/24	(3.9 ^a ± 0.2 x 10 ⁵)
	0.007	0/23	(6.0 ± 0.8 x 10 ⁴)
35°C	0.7	2/24	(1.7 ± 0.2 x 10 ⁵)
	0.007	0/23	(1.9 ± 0.2 x 10 ⁴)
37°C	0.7	6/24	(1.1 ± 0.1 x 10 ⁴)
	0.007	0/24	(4.7 ± 0.9 x 10 ³)

^a Mean titer (PFU/0.2 ml) ± standard error of the mean.

contained upon harvest only small plaque, non-revertant virus. At the higher temperatures of 35°C and 37°C, cell cultures inoculated with the 0.7 MOI contained large plaque virus which also formed plaques at 39.3°C in plaque assays at this temperature. The percentage of cultures with revertant, large plaque virus increased from 8% at 35°C to 25% at 37°C. In contrast, the cell cultures inoculated with the relatively low (0.007) MOI and incubated at 35°C and 37°C contained no large plaques in the 7 day harvests. The inability to detect large plaque virus in these harvests may be attributed to the lower yields obtained as a result of the low input inoculum. However, there is only an approximate two-fold difference in titers of the harvests from 0.7 and 0.007 MOI inputs that were grown at 37°C, and the difference probably does not account for failure to observe revertant virus in the virus harvests from the low input cultures.

One proposal that may explain the observed temperature-dependent reversion of S-1 is a direct effect of temperature on the viral polymerase, resulting in a faulty transcript. A mistake in the transcript may be a suppressor mutation that corrects for the original mutation conferring temperature sensitivity. The result of this would be emergence of a population of large plaque, temperature resistant virus, similar to that found in parent virus stocks.

C. S-1 vaccine virus, RNA synthesis. Some ts mutant viruses do not synthesize RNA (or DNA) at their non-permissive temperature. These are characterized as RNA- while those mutants that are capable of synthesizing viral-specific RNA to levels comparable to the wild type virus are labeled RNA+. A method for determining the RNA[±] characteristics of measles mutants has been described by Haspel and Rapp, 1975 (2), employing a shift of virus-infected cultures from non-permissive to permissive temperature in the presence of 5-azacytidine (5-AC). The 5-AC is a mutagen which inhibits production of fully functional RNA by incorporation as an analog. Only previously synthesized RNA would result in the production of infectious virus after shifting to permissive temperature, since 5-AC would inhibit newly-synthesized RNA from being functional. Virus titers after shift are compared to parent virus titers (assuming the parent is RNA+) to determine RNA[±] characteristics.

Table 2 lists the results of such a shift-down experiment using S-1 and GM-6 (parent) viruses. S-1 infected FRhL cultures were held 4 days at 38.5°C while GM-6 infected cultures were held for the same period of time at 39.3°C. At the time of shift, 5-AC was

Table 2. RNA synthesis of S-1 and GM-6 viruses after shift to permissive temperature in the presence of 5-AC.

Virus	Days post shift	5-azacytidine ($\mu\text{g/ml}$)		
		0	10	25
S-1 (held at 38.5°C for 4 days and shifted to 35°C)	1	1.3×10^3 ^a	1.1×10^3 (85%)	2.5×10^2 (19%)
	2	4.9×10^2	1.6×10^3 (326%)	3.6×10^2 (73%)
GM-6 (held at 39.3°C for 4 days and shifted to 35°C)	1	2.0×10^3	6.6×10^2 (33%)	3.3×10^2 (17%)
	2	3.6×10^3	1.1×10^3 (31%)	3.8×10^2 (11%)

^a PFU/0.2 ml

added to the cell culture maintenance media at concentrations of 0, 10, 25, and 50 $\mu\text{g/ml}$ and left there through the remainder of the experiment.

Drug treated cultures are compared to cultures receiving no drug and virus titers are listed as a percent of the control. S-1 virus titers from cultures receiving 50 $\mu\text{g/ml}$ of 5-AC are reduced to 4% of the control two days following shift. GM-6 titers are similarly reduced by this concentration of drug. Assuming that we are measuring the product (virus) of infectious RNA formed at non-permissive temperature over the 4 day holding period and **that we are not** measuring newly synthesized RNA, then S-1 appears to be RNA+. Comparison is made with the GM-6 parent virus which we assume to be RNA+.

D. S-1 replication at non-permissive temperature. Experiments were undertaken to examine replication of the S-1 vaccine virus. The following points were studied: (1) ability of infected cells to produce virus after being shifted from non-permissive to permissive temperature will give information on initiation of infection (early stages of infection not counting adsorption), persistence at the non-permissive temperature, and spread of infection; (2) antigen production at the non-permissive temperature; antigen production without infectious virus production would indicate a block in a late stage of maturation of S-1 or in the release of this virus; (3) cell bound virus at non-permissive temperature; concentration of intracellular infectious virus would indicate some impairment of release of the S-1 virus.

Infectious center assays were used to study adsorption of virus, viral persistence, and spread of infection. S-1 and GM-6 virus replication was compared by infecting FRhL cells and at intervals post inoculation, cell cultures were trypsinized and aliquots of cells plaqued in LLC-MK₂ plaquing flasks. Infected FRhL flasks were held at either 35°C or 38.5°C for the appropriate intervals and plaque assays were done at 35°C. Table 3 lists the results of such an experiment where each data point represents the number of infectious centers, i.e. the number of infected cells that gave rise to plaques upon assay in the permissive LLC-MK₂ plaque assay. At 35°C, GM-6 infected nearly 100% of available cells after 3 days while S-1 at the same temperature was capable of infecting approximately 3% of the cells over the 3 day period. This lag observed for S-1 appears to begin immediately upon infection and is reflected in the virus titers for days 1 and 2 also. The 0 time point represents samples plated immediately following adsorption prior to any temperature shift and the

Table 3. Infectious center assays for S-1 and GM-6 viruses incubated at 35°C and 38.5°C.

Virus	Temperature	Day	Infectious centers/ 10^5 cells
S-1	35°C	0	$< 0.3 \times 10^1$
		1	0.9×10^1
		2	2.1×10^2
		3	3.8×10^3
	38.5°C	1	0.3×10^1
		2	$< 0.8 \times 10^1$
		3	$< 1.3 \times 10^1$
GM-6	35°C	0	7.3×10^1
		1	4.1×10^3
		2	1.6×10^4
		3	8.0×10^4
	38.5°C	1	2.2×10^2
		2	4.8×10^2
		3	2.1×10^3

difference observed between the infectious center titers for S-1 and GM-6 at this point may be indicating impaired adsorption of S-1 at 35°C. All adsorptions were done at 35°C which was thought to be permissive. The MOI for S-1 and GM-6 was 0.3 and 0.09, respectively. The low number of cells infected with S-1 virus is reflected in the inability to detect infected cells following adsorption. The impaired adsorption or penetration is also sensitive to temperature since no infectious centers were observed for 3 days in S-1 infected cultures held at 38.5°C. After 4 days (data not shown) infectious centers were seen in S-1 plated cells that had been incubated at 38.5°C. At this time, leaky virus was also seen in supernatant fluid culture samples, also.

Some ts mutants produce viral antigen at the non-permissive temperature without production of infectious virus. This usually indicates that viral proteins are translated and a late block in replication is preventing maturation of infectious virus. S-1 virus was compared to GM-6 virus for the capacity to make viral antigen at the non-permissive temperature of 38.5°C. Table 4 lists the results of a fluorescent antibody (FA) assay of viral

Table 4. Detection of viral antigen by indirect fluorescent antibody assay of S-1 and GM-6 infected cells.

Virus	Temp	Day post inoc	PFU/ml	FA foci/well (0.2 ml inoc)
S-1	35°C	1	0	5
		2	3.2×10^3	16
		3	2.9×10^5	64
	38.5°C	1	0	8
		2	5	31
		3	1.0×10^2	52
GM-6	35°C	1	2.3×10^3	3
		2	1.2×10^5	45
		3	7.5×10^5	44
	38.5°C	1	0	13
		2	3.7×10^3	16
		3	1.0×10^4	29

antigen for three consecutive days following infection. FA foci as well as infectious virus in the supernatant fluids were assayed from cell cultures held at 35°C and 38.5°C. FA foci were seen for S-1 at 38.5°C with a large reduction in infectious virus (day 2). Cells containing viral antigen in the form of fluorescent foci were present for the S-1 virus infected cells at 38.5°C in greater numbers than GM-6 infected cells at the same temperature. Infectious virus titers for GM-6 were at least 100 fold higher than S-1 at this temperature.

Experiments of this type indicate that the S-1 virus is capable of translating some, if not all, of its coded structural and non-structural proteins. The observed temperature sensitivity of this virus, i.e. reduced virus titers at non-permissive temperature, appears to result from blockage of a late viral function, possibly during maturation or release of infectious virions.

Release of virus can be studied by assaying intracellular virus and comparing titers to virus already released into the supernatant fluid culture medium. S-1 and GM-6 infected cells held for 3 days at both 35°C and 38.5°C were washed free of supernatant fluid virus and freeze-thawed 3 times to release intracellular virus. Virus resulting from the freeze-thaw was titrated along with supernatant fluid virus removed from the same cell culture. Table 5 lists the ratios of cell-bound to released S-1 and GM-6 viruses. The ratio for S-1 at 38.5°C is slightly higher than the ratio calculated for the 35°C held culture. In contrast to this, the ratio for GM-6 at 38.5°C is approximately 7-fold higher than the 35°C ratio. Thermal inactivation of supernatant fluid virus at 38.5°C may account for the higher ratio for GM-6, however both S-1 and GM-6 viruses appear to have similar thermolabilities. S-1 virus by this type of assay is being released from the infected cell at least as efficiently as GM-6 virus at 38.5°C and by all indications, the GM-6 virus release is being inhibited by the non-permissive temperature. Therefore, release of S-1 virus appears not to be affected by the temperature sensitive event.

Table 5. Ratios of cell-bound to released S-1 and GM-6 at permissive and non-permissive temperatures.

	intracellular PFU/sup fld PFU
S-1 @ 35°C, day 3	$2.5 \times 10^4 / 1.2 \times 10^5 = 0.21$
38.5°C, "	$6.0 \times 10^1 / 1.6 \times 10^2 = 0.37$
GM-6 @ 35°C, day 3	$5.1 \times 10^4 / 2.7 \times 10^5 = 0.19$
38.5°C, "	$3.2 \times 10^3 / 2.4 \times 10^3 = 1.3$

E. Passage of S-1 vaccine virus at 31°C. S-1 vaccine, lot #1 produced clinical symptoms in human volunteers without indications that the virus reverted during replication in the volunteer. A series of ten passages of S-1 was done at 31°C to lower the non-permissive temperature of this virus. A lower non-permissive temperature may inhibit replication of the virus resulting in a more limited infection and fewer clinical symptoms in human recipients.

Passages of S-1 were done at regular 7 day intervals at 31°C and harvests from each passage titrated in plaque assays done at 35°C and 38.5°C. EOP values (38.5°C PFU/35°C PFU) were calculated for each titration. In summary (details can be found in Annual Report, 1978, Department of Biological Research), passage 10 showed a significantly higher EOP value than did previous passages of the S-1 virus inoculum. There were also indications of the emergence of a larger plaque-size population by the 10th passage. These large plaques did not resemble those seen in titrations of the parent GM-6 virus. Suckling mouse IC LD₅₀ titers will also be compared for in vivo correlates of the observed increased temperature resistance of this virus.

II. Dengue-3 Vaccine Progress.

A. Purification and homogeneity of the C-5 clone. Using FRhL passage 10 of the C-5 clone, a series of three terminal dilution platings of the virus were initiated to insure the homogeneity of this clone. The C-5 clone was originally isolated at the passage 7 level by a terminal dilution plating of parent (CH53489) virus. For terminal dilution plating, the C-5 virus was diluted so that a small number of wells of a 24-well Linbro cell culture plate, containing FRhL cells, were infected. Supernatant fluid from each well was assayed by plaquing in LLC-MK₂ cells and a positive well with small plaque virus was used to initiate the next plating passage. Table 6 lists the results of the three consecutive terminal dilution passages. At the FRhL-13a passage (third of three), cover slips were added to the bottom of the Linbro plate wells so that fluorescent antibody (FA) antigen testing could be done. As seen in the results, the number of FA positive wells was 2/3 that of the number of wells containing infectious virus. Most of the FA positive wells contained single foci of fluorescing cells indicating good distribution of inoculum and a high chance of cloning a single population of virus.

B. Passage and characterization of C-5 sublines. Seven wells containing virus from the FRhL-13a passage (from plates receiving 6 and 2 PFU/well input) were used to establish sublines

Table 6. Terminal dilution platings of the C-5 clone.

Passage	Input PFU/well	No. of positive wells PFU	No. of positive wells (FA)
FRhL-11a	28	5/24	ND
-12a	30	2/24	ND
-13a	18	10/24	6/24*
	6	4/24	3/24
	2	3/24	2/24

* 8/11 wells with single foci.

2/11 wells with 3 foci.

1/11 wells with 12 foci.

of the C-5 clone. To make a final determination on the homogeneity of the C-5 clone virus, seed pools of the sublines were prepared at the FRhL-14a and FRhL-15a passage levels. In order to obtain a high enough MOI for productive infection, inoculation had to be done first in 25 cm² flasks, then in 150 cm² flasks at the 14a and 15a passages respectively.

For characterization of the sublines, newborn mice were inoculated with each of the sublines (FRhL-14a). Following inoculation, 2 mice from the pool that received each subline were sacrificed daily for recovery of virus from the brain. Table 7 lists the growth curves for each subline in mouse brain. Compared to the FRhL-6 parent which was also used for mouse inoculation, the C-5 sublines grew very poorly in mouse brain. All plaques from assays of the C-5 inoculated brain homogenates were small, while those from the FRhL-6 parent-inoculated mice were typically mixed.

Sublines 1, 4, and 5 were chosen for passage in FRhL cells at 35°C and 36.5°C to evaluate their genetic stability. The 35°C temperature is fully permissive while 36.5°C is partially non-permissive. From previous growth curves it was found that C-5 replication was completely shut off at 38.5°C. Table 8 lists the 14 day supernatant fluid virus titers for each subline at the two temperatures. The results include passage 16b and 17b; passage 18b and another repeat of passage 18 (18c) did not yield virus either at 35°C or 36.5°C. For the 2 passages from which we could harvest virus, there appeared to be no change in the 3 sublines, i.e. no emergence in the harvests of large plaque, temperature resistant virus. The 36.5°C temperature was included because it was previously found with the DEN-2, S-1 virus that a low non-permissive temperature induced reversion of this virus. C-5 sublines 1 and 5 grew to a low titer at this temperature and did not show evidence of reversion. Only subline 5 could be passaged a second time at this temperature and the 14 day harvest still contained small plaque virus. The C-5 vaccine will be prepared at the 17th passage level using subline 4. It appears that subline 4 will be stable through this passage level.

A growth curve of the C-5 subline 4 was done so that it could be compared to previous growth curves performed with this clone. Table 9 lists the results of a growth curve done at 35°C, 37°C, and 38.5°C using FRhL-16a passage virus as the inoculum. As in the past, growth at 37°C was minimal and growth at 38.5°C was shut off. Plaque morphology of each harvest sample from the 35°C and 37°C growth curves was small; there was no evidence of reversion or genetic change.

Table 7. Growth curves of C-5 sublines #1-#7 and FRhL-6 (parent) viruses in suckling mouse brain.

Input *	Day	Subline							FRhL-6 (parent)
		<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	
		<10 ¹	5.0 X 10 ¹	<10 ¹	1.5 X 10 ²	7.0 X 10 ²	1.3 X 10 ²	1.4 X 10 ²	6.0 X 10 ¹
1	**	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	1.0 X 10 ¹
3	0	0	0	3	0	0	0	0	2.2 X 10 ²
4	0	0	0	0	0	0	0	0	7.0 X 10 ²
5	0	0	0	0	6	0	0	0	4.4 X 10 ³
6	0	0	4	0	7	11	7	3	3.7 X 10 ²
7	0	0	3	0	11	6	2	0	7.2 X 10 ³
8	0	0	0	0	0	3	0	0	3.0 X 10 ²
9	0	0	0	0	5	50	0	0	3.5 X 10 ³
10	0	0	0	0	0	0	0	0	1.8 X 10 ³
11	ND	ND	ND	0	ND	0	ND	ND	2.0 X 10 ¹
12	ND	ND	ND	0	ND	4	ND	ND	<10

* Input inoc; PFU/0.02 ml given I.C.

** PFU/0.2 ml from 20% mouse brain homogenates.

Table 8. Passage of C-5 sublines 1, 4, and 5 at 35°C and 36.5°C in FRhL cells.

C-5 subline	Passage, Temp.	PFU/0.2 ml	
		35°C	39.3°C
Sub - 1	16b, 35°C	1.3×10^3 (small)	0
	36.5°C	2.1×10^2 (small)	0
- 4	16b, 35°C	2.4×10^3 (small)	0
	36.5°C	0	0
-5	16b, 35°C	1.9×10^3 (small)	0
	36.5°C	2.0×10^2 (small)	0
Sub - 1	17b, 35°C	1.9×10^3 (small)	0
	36.5°C	0	0
- 4	17b, 35°C	1.0×10^3 (small)	0
	36.5°C	0	
- 5	17b, 35°C	1.2×10^3 (small)	0
	36.5°C	9 (small)	0

Table 9. Growth curves of the C-5 subline 4 at 35°C, 37°C, and 38.5°C.

	PFU/0.2 ml ^a		
	35°C	37°C	38.5°C
Day 1	0	0	0
2	0	0	0
3	0	0	0
4	1	4	0
5	2.0×10^1	1.0×10^1	0
6	8.0×10^1	1.0×10^1	0
7	1.3×10^2	1.7×10^1	0
8	3.0×10^2	4.2×10^1	0
9	1.0×10^2	4.8×10^1	0
10	5.0×10^1	1.1×10^1	0

^a plaque titrations done at 35°C

Efficiency of plating (EOP) has been used as a measure of temperature sensitivity for both DEN-2 and DEN-3 clones. Plaque titer at non-permissive temperature is divided by the plaque titer at permissive temperature and the ratio is the EOP. Table 10 lists the EOPs for several different C-5 passage seeds calculated for plaque formation at 37°C, 38.5°C, and 39.3°C. The parent, FRhL-6 virus EOP, is also included as a point of comparison. As can be seen from the table, there was no significant increase in the EOP at 37°C or 38.5°C for C-5 as it was passed 6 consecutive times. An increased EOP at the 16th passage would indicate increased temperature resistance and growth characteristics similar to the parent virus.

Table 10. Comparative plaquing of C-5 clone virus at 35°C, 37°C, 38.5°C, and 39.3°C.

Virus	Passage	PFU/0.2 ml			
		35°C	37°C	38.5°C	39.3°C
C-5	FRhL-10	8.8×10^2	$5.6 \times 10^2 (0.64)^*$	$1.2 \times 10^1 (0.014)$	0
	14a	2.8×10^3	$1.7 \times 10^3 (0.61)$	$1.4 \times 10^1 (0.005)$	0
	15a	8.3×10^2	$6.6 \times 10^2 (0.79)$	4 (0.0048)	0
	16a	1.5×10^3	$1.3 \times 10^3 (0.87)$	$3.2 \times 10^1 (0.021)$	0
parent	FRhL-6	2.8×10^4	$1.6 \times 10^4 (0.57)$	$4.2 \times 10^3 (0.15)$	$5.1 \times 10^2 (0.018)$

* EOP for each temperature shown in parentheses.

C. Preparation of a master (working) seed for C-5. Subline 4 of the C-5 clone was chosen for further passage and preparation of a master seed. No significant difference was seen for any of the sublines in the tests just described. Subline 4 grew to slightly higher titer than the other sublines at 35°C during passage 15 in the FRhL cells and did not grow at 36.5°C in the genetic stability test. However, we felt confident that any one of the seven sublines could be used for further passage.

Roller flasks (490 cm²) containing FRhL cell monolayers were inoculated with undilute seed made at the 15th passage. Flasks were re-fed twice a week and harvests were done on days 14 and 21 post inoculation, respectively. By day 18, focal patches of CPE were observed in the inoculated flasks indicating growth of the C-5 virus. For each harvest, flasks were assayed individually for virus titers and plaque morphology. Virus harvests were kept at 4°C after mixing with an equal volume of fetal bovine serum for stability. The 21 day harvest was freeze-dried; the 14 day harvest appeared to be not as stable after the 4°C holding period and freezing at -70°C. Table 11 lists the mean titers over 35 days for the 3 flasks infected with the C-5 clone for the master seed preparation. Flasks were re-fed and sampled past the 21 day harvest to observe for evidence of reversion. No clear evidence of reversion was found. In some plaque assays occasional large plaques were seen, however this usually could not be repeated in additional plaque assays. Plaques at 39.3°C were not found in harvests through day 35.

The master seed was tested for bacteriological and mycoplasma contamination as well as for viral adventitious agents. Day 35 control flasks were tested for hemadsorbing agents using guinea pig red blood cells. Animal testing included suckling and adult mice, rabbits, and guinea pigs. None of the safety tests revealed the presence of either contamination or adventitious agents.

D. Monkey experiments using the C-5 clone. It was previously shown that the C-5 clone inoculated into rhesus monkeys did not produce a viremia, stimulated neutralizing CF and HI antibodies, and protected against challenge with the parent virus (Annual Report, 1977). Further experiments were done with rhesus and cynomolgus monkeys inoculated with the C-5, subline 4 (FRhL-15a) and parent (FRhL-6) viruses. As shown in Table 12, after 2 rhesus monkeys were inoculated with the C-5 clone, only one had a one day viremia while neither cynomolgus monkey, similarly inoculated, produced viremia. Both cynomolgus monkeys that received the parent virus produced viremia of one and two day duration. Viremias were assayed by direct plaque assay and also by a delayed

Table 11. Replication of C-5 subline 4 virus during the master seed passage.

Sample, day	PFU/0.2 ml	
	35°C	39.3°C
4	< 10	ND
7	4.6×10^2	ND
11	1.1×10^3	ND
14	2.1×10^3	ND
18	1.1×10^3	0
21	4.2×10^3	ND
28	1.3×10^3	0
31	2.3×10^3	ND
35	1.1×10^3	0

plaque assay. Direct assay consisted of plating undilute serum and overlaying directly. The delayed assay consisted of inoculating monolayers of LLC-MK₂ cells, incubating at 35°C for 2 weeks and plaquing the supernatant culture fluids from these flasks. As can be seen from Table 12, one day of viremia for monkey 528 was detected by the delayed technique but not by the direct assay. Further reliability of detecting viremia was seen using the delayed technique in assaying frozen serum from previously inoculated monkeys. Frozen (approximately 2 years at - 70°C) serum specimens for monkey 331 were assayed by delayed technique and virus found in specimens for 2 days. Previous assay by a direct technique did not detect this virus. Also included in Table 12 are data from 2 monkeys, 339 and 368, which were inoculated with the parent DEN-3 virus. Delayed assay detected virus in 3/10 serum samples for each monkey. The day of viremia found for the delayed versus the direct assays did not completely coincide.

Table 12. Rhesus and cynomolgus monkeys inoculated with the C-5 clone and parent DEN-3 viruses.

Monkey no.	Virus inoc.	Direct assay		Delayed assay	
		Days viremic		Days viremic	
526 (rh)	C-5:2.5 X 10 ³ PFU	0/12		0/12	
528 (rh)	"	0/12		1/12, day 5	
6608 (cyno)	"	0/12		0/12	
6680 (cyno)	"	0/12		0/12	
6322 (cyno)	parent: 1.3 X 10 ⁴ PFU	2/12, days 2, 3		2/12, days 2, 3	
6593 (cyno)	"	1/12, day 2		1/12, day 2	
		* * * * *			
331 (rh)	C-5: 3.0 X 10 ³ PFU	0/10		2/10, days 6, 7	
332 (rh)	"	0/10		0/10	
339 (rh)	parent: 4.0 X 10 ⁴ PFU	3/10, days 6, 7, 8		3/10, days 5, 6, 7	
368 (rh)	"	3/10, days 2, 5, 6		3/10, days 5, 6, 7	

DEN-3 virus titers in viremic monkey sera usually do not exceed 1 log when plaqued directly. In many cases, single or very few plaques are counted in daily serum specimens. The delayed plaque technique, although taking 3 times the amount of time to complete, appears to be more sensitive and detects virus where it is not detected by direct assay. This technique is especially useful when monkeys are inoculated with attenuated viruses where low levels of viremia are expected.

The virulence of the C-5 clone appears not to have changed upon passage in FRhL cells. Monkeys 331 and 332 received C-5 virus at the 8th passage level and monkeys 526, 528, 6608, and 6680 were inoculated with passage 15 virus. These results are in full agreement with suckling mouse and cell culture growth curve data. Table 13 lists the antibody responses of these monkeys. All monkeys were infected and stimulated production of CF, HI, and neutralizing antibodies. Antibody titers are comparable to those titers found in monkeys receiving the DEN-3 parent virus.

Table 13. Antibody responses of monkeys inoculated with the C-5 clone and parent DEN-3 viruses.

Monkey no.	Reciprocal antibody titer on day						
	CF			HI			Neut
	15	30	45	15	30	45	
526	32	128	32	80	160	320	410
528	64	64	32	160	320	160	800
6608	32	64	16	320	160	80	820
6680	16	32	4	80	40	20	190
6322	32	64	16	160	160	160	960
6593	256	128	64	640	320	320	790

E. C-5 antigen production in mouse brain. Crude suckling mouse brain preparations contain non-specific substances which fix complement. Supernatants from twenty per cent mouse brain homogenates, centrifuged at 2,000 rpm for 20 min were used to compare CF antigen production in mice inoculated intracerebrally with C-5 and parent DEN-3 viruses. It was found that mock-inoculated mouse brain homogenates fixed complement along with

the virus-inoculated homogenates in CF tests. The non-specific factors could be removed by final centrifugation at 10,000 rpm for 1 hr. The supernatant fluid fractions were assayed for CF antigen with DEN-3 mouse hyperimmune ascitic fluid or monkey antisera raised against a single inoculation with the DEN-3 parent virus. The CF and PFU titers are listed in Table 14 for each day following inoculation with the C-5 and parent viruses.

CF antigen is found early (days 1 and 2) for both C-5 and parent-inoculated mice when tests are run using the monkey antisera. CF antigen is also found later in mice receiving both viruses when mouse ascitic fluid is used as a reagent in the CF test. CF antigen found on days 6, 7, and 8 in mice inoculated with the parent virus appears to coincide with infectious virus also found at approximately the same time. However, the CF antigen found on day 5 post inoculation with the C-5 clone does not coincide with infectious virus recovered from the same mouse brain sample. Further characterization of the antigens found in these preparations is necessary to understand the observed results of these experiments.

Table 14. Complement-fixing antigen and infectious virus from mouse brain preparations of mice inoculated with the C-5 clone and parent DEN-3 viruses.

	C-5 clone			parent			mock		
	PFU/0.2 ml	CF (mouse ab)	CF (mk ab)	PFU/0.2 ml	CF (mouse ab)	CF (mk ab)	PFU/0.2 ml	CF (mouse ab)	CF (mk ab)
Day 1	0	< 2	16	0	< 2	4	ND	< 2	< 2
2	0	< 2	2	4	< 2	4	ND	< 2	< 2
3	5	< 2	< 2	1.5×10^1	< 2	< 2	ND	< 2	< 2
4	1.4×10^1	< 2	< 2	4.3×10^2	< 2	< 2	ND	< 2	< 2
5	0	16	< 2	1.3×10^3	< 2	< 2	ND	< 2	< 2
6	3.2×10^1	< 2	< 2	5.0×10^2	8	< 2	ND	< 2	< 2
7	1.0×10^1	< 2	< 2	3.0×10^4	16	< 2	0	< 2	< 2
8	0	< 2	< 2	3.3×10^2	8	< 2	ND	< 2	< 2
9	0	< 2	< 2	5.0×10^1	< 2	< 2	ND	< 2	< 2
10	0	< 2	< 2	2.0×10^2	< 2	< 2	ND	< 2	< 2
11	0	< 2	< 2	1.4×10^2	< 2	< 2	ND	< 2	< 2
12	0	< 2	< 2	< 10	< 2	< 2	ND	< 2	< 2
13	0	< 2	< 2	< 10	< 2	< 2	ND	< 2	< 2
14	0	< 2	< 2	< 10	< 2	< 2	0	< 2	< 2

Project 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 014 Characteristics of attenuated dengue viruses

Literature Cited.

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(U) Animal Models; Schistosomiasis; (U) Drug Development; (U) Antiparasitic							
35. TECHNICAL OBJECTIVE, 36. APPROACH, 37. PROGRESS (Pursuit individual paragraphs identified by number. Provide rest of each with Security Classification Code.)							
<p>23. (U) To find new drugs with chemoprophylactic or chemotherapeutic activity against <i>Schistosoma mansoni</i>, which can be used by military personnel to prevent or treat the disease in endemic areas.</p> <p>24. (U) Compounds previously shown to be active in screening tests will be re-examined to determine their optimum prophylactic and/or therapeutic treatment regimens in the mouse and/or subhuman primate models. Prophylactic agents for either topical application or systemic administration are being developed. Available chemical analogues will be tested and efforts will be made to determine the relationships between chemical structures, modes of antiparasitic action and modes of toxicity. Analogues with increased therapeutic efficacy and decreased host toxicity will be identified, and where justified, submitted for preclinical studies.</p> <p>25. (U) 77 10 - 78 09. During this interval chemicals were screened topically and/or systemically in the ICR mouse model. Three compounds screened topically in the primary prophylactic test prolonged survival of mice exposed to 3,000 cercariae. Thirty-six compounds screened systemically failed to provide prophylaxis in this test system. Of the fifty-seven compounds screened topically in the secondary prophylactic test, five decreased worm burdens only if the treated skin remained unwashed and twenty-one provided prophylaxis even following water washes of one or more hours. The three compounds screened systemically in the primary curative test reduced worm burdens below control levels. For Technical Report see Walter Reed Army Institute of Research Annual Report, 1 Oct 77-30 Sep 78.</p>							

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1418

PROJECT 3M162770A802 MILITARY PREVENTIVE MEDICINE

Work Unit 015 Chemotherapy and Chemoprophylaxis of Schistosomiasis

Investigators

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LTC David E. Davidson, Jr., VC

1. Description

Schistosomiasis is a debilitating parasitic disease currently afflicting an estimated 250 million people world-wide. During World War II 1500 U.S. Army personnel became infected during operations in the Philippines. Had the Viet Nam police action involved additional operations in parts of Laos and Cambodia, U.S. forces may have been infected with one of the species of parasitic worms causing this disease. Should our forces in the future be committed to areas of South America, the Carribean, Africa, the Middle East, or various parts of the Far East where this disease is endemic, they may be exposed to infection with one or more of the four major human schistosomes.

Currently available therapeutic drugs provide only partial cures and treatment is always associated with the risk of severe side effects. In addition, no safe but effective prophylactic drugs or vaccines are currently available for use by American military personnel. For these reasons, research is being conducted at WRAIR to discover and evaluate new prophylactic and curative drugs.

2. Progress

A. Prophylactic Drug Screen

In searching for prophylactic drugs, this laboratory has been examining the ability of drugs applied topically and/or systemically to prevent infection. In the topical test, compounds are dissolved in methanol, ethanol, or dimethyl sulfoxide at 5% or lower drug concentrations. The tails of conscious, restrained mice are immersed into 4 to 4.5 ml aliquots of this drug solution for a period of five minutes. The tails are subsequently air-dried. The tails of some mice are then washed in running tap water

for approximately thirty minutes at some time prior to cercarial exposure, and again air-dried. Twenty-four hours after the drug application, the mice are individually exposed by tail immersion to approximately 100 Schistosoma mansoni cercariae (WRAIR Puerto Rican strain) dispersed in 4 ml of dechlorinated tap water. Forty-nine days later the mice are sacrificed by sodium pentobarbital injection, their worms recovered by perfusion of the portal circulation, and the worms counted. The mean worm burden from each drug treated test group is then compared to the mean worm burden of the control group treated with the drug solvent solution. Compounds are considered active if unpenetrated cercarial bodies are found in the immersion suspension tube and/or if most worm burdens are reduced by at least fifty percent. Table 1 presents the number of compounds of various chemical classes tested topically and the number of active members. As can be seen from this table, a total of sixty-six compounds were tested during this fiscal year. Thirty-three compounds were active when applied one day prior to cercarial exposure. Of these, twenty-three compounds were not removed by the water wash. The 4-aminoquinoline, 8-aminoquinoline, and biphenyl derivative classes contained the most active compounds. Of the latter class, bis(3,5,5-trichloro-2-hydroxyphenyl) methane (WR 22537), bis(5-chloro-2-hydroxyphenyl) methane (WR 3924), bis(3,5-dichloro-2-hydroxyphenyl) sulfide (WR 17018), 4-nitro,4'-isothiocyanate-diphenylamine (WR 234927), and 4'-isothiocyanate-dibenzyl ether (WR 234928) appeared to be the most active compounds. Data indicating the protection afforded by prior treatment with various concentrations of these compounds are presented in Table 2. These values are for mice subjected to the water bath wash subsequent to drug treatment and before cercarial exposure.

In the evaluation of drugs for prophylactic activities following systemic administration, two kinds of tests are conducted. In the primary systemic prophylactic test, drugs are dissolved or suspended in a saline solution containing 0.2% (v/v) methyl cellulose plus 0.4% (v/v) Tween 80, or in a 20% (v/v) aqueous Cremophor EL vehicle. They are administered to the mice by subcutaneous injection one day prior to their exposure to 3,000 cercariae. The effectiveness of the drug treatments are evaluated in terms of increases in survival times of the treated versus untreated mice. If mice from any group survive to day forty-nine post infection, they are necropsied, their worms recovered by venous perfusion, and the worms counted. Niridazole

is used as a positive control drug of known high prophylactic activity. Drug treatment is similar in the secondary prophylactic test. In the secondary test, however, mice are exposed to 100 cercariae. Their worms are then recovered by venous perfusion on day forty-nine post-infection. Worm burdens of drug treated and drug solvent treated mice are then compared.

During this fiscal year, seventy-five compounds were tested systemically. Table 3 presents the number of compounds of each class which were tested. Only three new compounds demonstrated significant activities. The most active was 2(p-chlorophenyl)-alpha-2-piperidyl-4-quinolinemethanol (WR 7929). An acridine (WR 2904) and a second quinoline-methanol (WR 29252) also demonstrated activities. All three compounds were more active than niridazole in this test model.

B. Studies on Hexachlorophene as a Topical Antipenetrant

As hexachlorophene had been previously shown to exhibit high antischistosomal activity in the topical test, additional studies were conducted to further evaluate its potential usefulness as a prophylactic agent. Studies have been conducted to determine the duration of protection offered by a single skin treatment with 1.25% (w/v) hexachlorophene in methanol. Table 4 presents the results from this study. Here useful protection against exposures to approximately 100 cercariae is indicated for intervals of up to three days. Statistically significant protection, however, extended to ten days post-treatment. In addition, the resistance of hexachlorophene to removal by water washing treatment has been determined. This data is presented in Table 5. Although some protection was lost with washes as short as three hours, very substantial prophylactic activity was still present even after washes of five hours duration.

Studies were also conducted to determine if hexachlorophene's activity could be attenuated by subsequent treatment of skin with diethyl ether or diethyl ether containing 20% (w/v) linoleic acid. Linoleic acid is a lipid known to induce *S. mansoni* cercarial penetration responses. It was reasoned that hexachlorophene might act by either extracting or chemically altering mouse skin lipids, thereby removing the normal stimuli for penetration. In this study, both the number of cercariae which failed to penetrate and the number which developed into adult worms were examined.

Penetration failure was measured by the number of cercariae or cercarial bodies which remained in the exposure tubes at the end of the exposure period. Table 6 presents the percentages of cercariae which failed to penetrate the tail skin of the mice in each of the experimental groups. Here, hexachlorophene treatment was followed by air drying and then washing for three hours in running tap water. It was clearly shown that hexachlorophene inhibited cercarial penetration and that its activity was not significantly removed by subsequent ether treatment. 20% (w/v) linoleic acid treatment alone was shown to be inhibitory to cercarial penetration. Data in Table 6 suggest that linoleic acid treatment of hexachlorophene treated skin reduced the latter's antipenetrant activity. The validity of this conclusion is, however, challenged by the results on adult worm recoveries presented later. Finally, treatment of mouse tail skins with lipid solvents like methanol or diethyl ether one day prior to exposure does not appear to reduce cercarial penetration success from values routinely observed with untreated control mice.

Table 7 presents for the above study the percentage of the cercarial dosage recoverable as adult worms on day 49 post-exposure. Hexachlorophene treatment nearly prevented infection in all mice. Linoleic acid treatment at 20% (w/v) very significantly reduced worm burdens from levels observed in solvent treated controls. Perhaps treatment of skin with high concentrations of this lipid caused cercariae to become disoriented during their penetration efforts. Such cercariae may adhere to the mouse skin rather than penetrate it. This may explain why most cercariae for this group are not recoverable in the infection tubes or subsequently as adult worms from the veins of exposed mice. Further work on this aspect is needed.

As some linoleic acid treatments have been shown to inhibit cercarial penetration, the effects of graded concentrations of linoleic acid on cercarial penetration success have been examined. Table 8 presents the results of this study. Treatment of tail skin with 20% (w/v) linoleic acid in diethyl ether or in methanol, with 2% (w/v) linoleic acid in methanol, or with 1% (w/v) linoleic acid in methanol are seen to be inhibitory. Treatment with 0.5% (w/v) or 0.2% linoleic acid in methanol are not clearly inhibitory. During this next fiscal year the resulting worm burdens of mice in these mouse groups will be determined. It will also be determined if a suitable concentration of linoleic acid will

- make hexachlorophene treated skin more susceptible to cercarial penetration.

Additional studies in progress include the examination of hexachlorophene treated skin following exposure to several thousand *S. mansoni* cercariae. It is hoped to determine if hexachlorophene can kill larvae which have successfully penetrated into the skin.

TABLE 1. CLASSES OF COMPOUNDS TESTED TOPICALLY FOR ANTI-SCHISTOSOMAL ACTIVITY AND NUMBERS OF COMPOUNDS SHOWING ACTIVITY

<u>Class</u>	<u>Number of Compounds Tested</u>	<u>Number of Compounds Active</u>	<u>Number of Compounds Active After Water Wash</u>
Methanolic plant extracts	19	3	1
4-Aminoquinolines	5	4	3
8-Aminoquinolines	5	4	2
Quinolinemethanols	4	2	1
Biphenyls	13	11	9
Imidazoles	2	2	2
Long alkyl chains	5	3	1
Miscellaneous aryl compounds	7	2	2
Dialkyl-tin / nucle- oside complexes	4	0	0
Dehydroabiestic acid analogs	2	2	2
TOTALS	66	33	23

TABLE 2. PERCENT SUPPRESSION OF SCHISTOSOMA MANSONI WORM BURDENS,
BY PROPHYLACTIC TOPICAL APPLICATION OF VARIOUS BIPHENYL COMPOUNDS *

Compounds	Concentration of Applied Compounds				
	2.5%	1.25%	0.625%	0.312%	0.156%
bis(3,5,6-trichloro-2-hydroxy-phenyl) methane (common name hexachlorophene)	---	100	100	97.3	86.3
bis(5-chloro-2-hydroxy-phenyl) methane	95	74	81	65	18
bis(3,5-dichloro-2-hydroxy-phenyl) sulfide (common name bithionol)	---	100	---	75	---
4-nitro, 4'-isothio-cyanate-diphenylamine	---	---	100	---	90
4-nitro, 4'-isothio-cyanate-dibenzyl ether	---	---	100	---	83

* Compounds were applied to the tail skin approximately twenty-four hours prior to cercarial exposure. Tails were water washed for thirty minutes between compound application and cercarial exposure. Compound concentrations are given in percent weight/volumes.

TABLE 3. COMPOUNDS TESTED SYSTEMICALLY FOR PROPHYLACTIC ANTI-SCHISTOSOMAL ACTIVITY IN THE MOUSE MODEL

<u>Compound Class</u>	<u>Number of Compounds Tested</u>	<u>Number of Active Compounds</u>
Quinolinemethanols	5	2
Phenanthrenemethanols	1	0
4-Aminoquinolines	22	0
8-Aminoquinolines	1	0
Miscellaneous quinolines	5	0
Acridines	6	1
Biphenyls	6	0
Dehydroabietic acid analogs	4	0
Thiazoles and Imidazoles	2	0
Miscellaneous aryl compounds	19	0
Aminothiols	4	0
	—	—
TOTALS	75	3

TABLE 4. PERCENT OF CERCARIAL DOSAGE* RECOVERABLE AS ADULT WORMS FROM MICE EXPOSED TO CERCARIAE AT DIFFERENT TIMES POST-HEXACHLOROPHENE-TREATMENT** OR POST-METHANOL-TREATMENT

Interval Between Topical Application and Cercarial Exposure	Hexachlorophene Treated	Methanol (Vehicle) Treated
1 Day	0.0 \pm 0.0*** (n=24)	35.2 \pm 4.6 (n=23)
3 Days	1.1 \pm 0.1 (n=20)	35.0 \pm 4.4 (n=20)
7 Days	13.1 \pm 5.5 (n=10)	31.2 \pm 5.5 (n=10)
8 Days	18.1 \pm 3.6 (n=24)	37.0 \pm 5.0 (n=13)
10 Days	21.8 \pm 3.8 (n=8)	31.1 \pm 5.6 (n=9)

*Exposures were to approximately 100 cercariae.

**Hexachlorophene treatments were 1.25% (w/v) hexachlorophene in methanol for five minutes. Treatment was followed by a water wash.

***Values are given as mean percent \pm 2 standard errors. n is the number of mice.

TABLE 5. THE PERCENT PROTECTION AGAINST CERCARIAL EXPOSURE*
 AFFORDED MICE BY SKIN TREATMENT WITH 1.25% (w/v) HEXACHLOROPHENE
 FOLLOWED BY WATER WASHES OF VARYING DURATION.

<u>Duration of Wash in Hours</u>	<u>Percent Protection</u>	<u>Experimental Group Size</u>
0	100	19 methanol; 20 hexachlorophene
1	100	19 methanol; 20 hexachlorophene
2	99.7	18 methanol; 20 hexachlorophene
3	87.0	10
4	92.1	19 methanol; 20 hexachlorophene
5	77.0	9 methanol; 10 hexachlorophene

* Individual mice exposed to approximately 100 cercariae.

TABLE 6. PERCENT CERCARIAL PENETRATION FAILURES FOLLOWING VARIOUS CHEMICAL TREATMENTS OF MOUSE TAIL SKIN.

<u>Treatment Group</u>	<u>Mean</u>	<u>Number of Mice</u>
1.25% hexachlorophene in MeOH	57.6	10
1.25% hexachlorophene in MeOH then diethyl ether	58.2	7
Diethyl ether	0.95	10
1.25% hexachlorophene in MeOH then 20% linoleic acid in diethyl ether	34.6	9
20% linoleic acid in diethyl ether	10.5	10
MeOH	1.45	10

TABLE 7. PERCENT CERCARIAL DOSAGE* RECOVERABLE AS ADULT WORMS
FORTY-NINE DAYS POST-EXPOSURE FOLLOWING VARIOUS CHEMICAL TREATMENTS
OF MOUSE TAIL SKIN.

<u>Treatment Group</u>	<u>Mean</u>	<u>Number of Mice</u>
1.25% hexachlorophene in MeOH	1.4	10
1.25% hexachlorophene in MeOH then diethyl ether	0.5	9
Diethyl ether	55.0	4
1.25% hexachlorophene in MeOH then 20% linoleic acid in diethyl ether	0.2	10
20% linoleic acid in diethyl ether	6.7	10
MeOH	52.0	8

* Mice exposed to 200 cercariae

TABLE 8. PERCENT CERCARIAL PENETRATION FAILURES FOLLOWING DIFFERENT LINOLEIC ACID TREATMENTS OF MOUSE TAIL SKIN.

<u>Treatment Group</u>	<u>Mean</u>	<u>Number of Mice</u>
20% linoleic acid in diethyl ether	10.5	10
20% linoleic acid in MeOH	11.5	4
2% linoleic acid in MeOH	18.2	10
1% linoleic acid in MeOH	10.1	5
0.5% linoleic acid in MeOH	4.9	5
0.2% linoleic acid in MeOH	1.8	10
MeOH	3.4	10

MALARIA PROPHYLAXIS

7

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DES'N INSTR ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS ^a	10. LEVEL OF GUM ^a
77 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. UDD UNIT
11. NO. / CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
A. PRIMARY		62770A		3M162770A803		00	
B. CONTRIBUTING		CARDS 114F				083	
12. TITLE (Precede with Security Classification Code) ^a							
(U) Protective Immunity in Protozoan Diseases							
13. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
002600 Biology 010100 Microbiology							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. FUNDING AGENCY		17. PERFORMANCE METHOD	
74 07		CONT		DA		C. In-House	
18. CONTRACT, GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
A. DATES/EFFECTIVE: NA				B. PRESENT		C. FUND (in thousands)	
B. NUMBER ^a				FISCAL YEAR		78	
C. TYPE				CURRENT		5.0	
D. KIND OF AWARD				79		7.0	
E. AMOUNT				637		678	
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMER ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research			
ADDRESS ^a Washington, DC 20012				Division of CD&I			
				ADDRESS ^a Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. and/or non-U.S. nationality)			
NAME ^a Garrison Rapmund, COL				NAME ^a Carter L. Diggs			
TELEPHONE ^a (202) 576-3551				TELEPHONE ^a (202) 576-3544			
23. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
24. REVISIONS (Precede with Security Classification Code) ^a							
(U) Antigens; (U) Protozoa; (U) Immunity; (U) Tropical Medicine; (U) Antibodies							
25. TECHNICAL OBJECTIVE ^a 26. APPROACH 27. PROGRAM (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23 (U) The objective of this work unit is to elucidate the protective mechanisms involved in immunity to malaria and African sleeping sickness. Malaria is a disease which has repeatedly impeded military operations and African sleeping sickness has a high potential for doing so should there be troops in the endemic area.</p> <p>24 (U) The approach used in these studies is to study in both animal models and through the use of in vitro techniques the response elicited by the immune system, to determine the roles of cellular and molecular mediators in these processes, and to design experimental immunogens which will provide the basis for future vaccine development programs.</p> <p>25 (U) 77 10 78 09 Studies on effector mechanisms against African trypanosomes indicate antibody dependent killing of these organisms. Two separate killing mechanisms have been demonstrated: (1) the alternate complement pathway can be activated by the antibody sensitized trypanosomes resulting in death of the organisms, and (2) cells can act as the cytotoxic mediator, again in collaboration with antibody. Evidence suggesting macrophage activation during trypanosomiasis has also been obtained. A number of surface coat antigens occur with high prevalence and may serve as appropriate targets for vaccine development. Tsetse flies have been infected with a recent human isolate and new antigenic variants have been isolated from mice infected by these flies. In other studies, artificially induced immunity against rodent malaria has been transferred to non-immune recipients with lymphoid cells. For technical report see Walter Reed Army Institute of Research Annual Progress Reports, 1 Oct 1977 - 30 Sep 1978.</p>							

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1432

Project 3M162770A803 MALARIA PROPHYLAXIS

Work Unit 083 Protective Immunity in Protozoan Diseases

Investigators:

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Teresa Jareed, Karen McDonough, James K. Lovelace,
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Williams, Donald Wong, Ph.D.

I. Studies on the prevalence of several antigenic types of Trypanosoma rhodesiense.

A. Antigenic type analysis of Trypanosoma rhodesiense isolates from humans in the Lambwe Valley Area of Kenya.

Objective: This study was designed to provide a preliminary estimate of the prevalence of known antigenic types of T. rhodesiense in an endemic area of Kenya.

Description: The property of African trypanosomes to undergo antigenic variation poses a major obstacle to production of an effective vaccine. One potential solution to this problem is the use of a polyvalent vaccine having specificity for several different antigenic types of trypanosomes. This approach to a vaccine would require immunization with antigens representing the antigenic types of trypanosomes most likely to occur during the initial parasitemia in the host, i.e., the antigenic types injected by the fly and those occurring by antigenic variation during the first wave of parasitemia. To determine the feasibility of this approach, more data is needed on the number and antigenic type of trypanosomes occurring in the host and the prevalence of different antigenic types in endemic areas. To obtain an estimate of the prevalence of defined antigenic types of trypanosomes, analysis of a series of recent human isolates of T. rhodesiense from the Lambwe Valley was undertaken. For this purpose, the variant specific immunofluorescence assay described in last year's report was used. The assay allowed the detection of 13 different antigenic types of T. rhodesiense each designated as a WRATat (Walter Reed Army Trypanozoon antigen type).

Progress: The immunofluorescence assay was used to determine the presence of the 13 defined antigenic types in 26 human isolates of *T. rhodesiense*. The results of this assay for isolates obtained during 1974-75 are shown in Table 1. The percentage of trypanosomes giving a positive reaction with each WRATat specific antiserum is shown, based on a count of 10,000 organisms per serum. Isolate number 18, from which WRATat 1 was originally obtained, was in fact 98 percent WRATat 1. No other antigenic type was detected. Isolate 3 and 16 had no trypanosomes reactive while other isolates showed a range of reactivity.

Some antigenic types were found much more frequently than others, e.g., WRATat 12 occurred in 12 of the 15 isolates assayed. In contrast, WRATat 1, 3 and 6 were each detected in only one isolate. WRATat 5 was not detected in any isolate assayed.

The total percentage of the trypanosomes in each isolate which were identified as WRATats was variable. Isolate 3 and 16 contained no WRATats while isolates 10 and 18 contained 92 and 98 percent WRATats respectively.

The results of the analysis of trypanosomes isolated in 1976-77 are shown in Table 2. Here as in Table 1, it is evident that some antigenic types were found more frequently than others. WRATats 2 and 12 were detected in 7 and 8 of the isolates, respectively, while WRATat 1 and 8 were each found in only one isolate. Also, as before, there was considerable variation in the total percentage of trypanosomes reactive with anti-WRATat sera. Isolate number 33 contained no detectable WRATats while number 34 and 36 had 97 and 86 percent trypanosomes found to be WRATats.

Discussion: With only thirteen antigenic types defined, greater than 25 percent of the parasites obtained from patients at various stages of infection could be typed. Also, on the basis of these data, no major changes in antigenic types present were seen between 1974 and 1977 suggesting little or no antigenic drift in the prevalent trypanosomes. Although these data are encouraging, there is a need for the development of additional defined antigenic types and continued isolation of trypanosomes from humans in the Lambwe Valley. Only after a more extensive analysis will it be possible to determine the potential for a polyvalent vaccine.

B. Variable Antigenic Type (VAT) specificity of sera from trypanosome infected humans in the Lambwe Valley.

Table 1

Results of VAT analysis of *T. rhodesiense* isolates obtained in 1974-1975 from humans in the Lambwe Valley area of Kenya

Human Isolate Number	1 ^c	Percentage Positive ^a													Calculated Total % Reactive
		2	3	4	5	6	7	8	9	10	11	12	13		
1		.6	.2	9.5			1	.4			5	1.5		12	
2		6.5		.1			.6	3		3.5	.4			17	
3														0	
5		3		4				6.5		2	8	.4		24	
7				3.1						.1		27		30	
9												.2		.2	
10									1		89	2	.1	92	
11									.3			22.5		23	
12				1			7.5		1.5			3		12	
13							.5		2			23		26	
16					1									0	
17		10					3.5	.3		9	1.5	.1		14	
18	98													98	
19		10		.2						5	.1	.1		15	
20				.3								41		41	

^a Percentage of the trypanosomes in each isolate giving a positive reaction with VAT specific rabbit antisera in an indirect fluorescent antibody assay. Each percentage is based on a count of 10,000 trypanosomes.

^b All isolates were obtained from different patients with rhodesian sleeping sickness.

^c WRATat specificity of rabbit antisera.

Table 2

Results of VAT analysis of *T. rhodesiense* isolates obtained in 1976-1977 from humans in the Lambwe Valley area of Kenya

Human Isolate Number	1 ^c	<u>Percentage Positive^a</u>													Calculated Total % Positive
		2	3	4	5	6	7	8	9	10	11	12	13		
21	.1													.1	
25					.2		.1			1.5		17	.2	.19	
26		.3	.4								4.5		3	.8	
27				7.5			.2							.8	
31						.1			.2			.5		.8	
32		4.4				.2					12	3.7	.8	.21	
33														0	
34		16			32						.4	49		.97	
36		.7						11		.9	3	70	.5	.86	
37		.2								13		.1		.13	
38												.1	.1	.2	
49		.2									.2	.1		.7	
50		.1	.3	.2					.1		.1		.3	1.1	

^a Percentage of the trypanosomes in each isolate giving a positive reaction with VAT specific rabbit antisera in an indirect fluorescent antibody assay. Each percentage is based on a count of 10,000 trypanosomes.

^b All isolates were obtained from different patients with rhodesian sleeping sickness.

^c WRATat specificity of rabbit antisera.

Objective: To determine the prevalence of WRATat specific antibody in the sera of sleeping sickness patients in the Lambwe Valley.

Description: An assay has been developed which allows a limited antigenic characterization and quantification of T. rhodesiense parasites from an endemic area of Kenya. This is an indirect fluorescent antibody assay using dried thin blood films as the antigen. Although the assay system is extremely sensitive, it provides only the capability to determine the variable antigenic type (VAT) of the trypanosomes present at the time of sample collection. The assay does not provide the ability to determine the host's experience with other VATs during the course of disease prior to sample collection. Pending the development of VAT specific RIA or ELISA tests, a lytic assay using intact, viable trypanosomes was used. This assay allowed the detection of antibody to known VATs of trypanosomes in the serum of a chronically infected host, and provided the most direct approach to determining the incidence of certain antigenic types in an endemic area. From this data the most prevalent VATs could be determined.

Sera from eleven different patients in the Lambwe Valley with diagnosed sleeping sickness were tested for lytic activity against suspensions of intact trypanosomes of each WRATat. The results of this assay are shown in Table 3. Complete or partial lysis is indicated by + and + respectively. All sera showed reactivity with 2 or more of the WRATats. Two sera, LVH 35 and 36 were reactive with 13 of the 16 antigenic types. As found in the IFA assay, some WRATats specificities were found more frequently than others. Of the 11 sera tested, 9 sera reacted with WRATat 6 and 8 sera reacted with WRATat 9. Specificity for other antigenic types was found less frequently.

Discussion: The results of this study show that infected humans in the Lambwe Valley have antibody to trypanosome antigen types 1 through 16. As the lytic assay used has previously been shown to be highly specific in this system, the results also indicate that each patient whose serum was assayed, had experienced one or more of the WRATats during the course of infection. Also, some of the antigenic types appear to have occurred in most of the patients tested. More data is necessary before conclusions can be drawn as to the significance of these common antigenic types. A much more extensive survey of the Lambwe Valley is needed, to include serum collection from both trypanosome infected and noninfected humans along with some limited clinical history. Also, additional defined antigenic types of trypanosomes must be

Table 3
Anti WRATat reactivity of human sera in
the lytic assay^a

Human Sera ^b	1 ^c	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	W
LVI 27		+		+	+	+	+	+	+			+				+	
28						+											
29			+	+													
30	+		-			+			+								
32														+			
33						+			+								
35	+	+	+	+	+	+	+	+	+		+		+	+	+	+	
36	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
51	-	+	+	+	+	+	+	+	+			+	+	+	+	+	+
52		+				+	+	+	+			+	+	+	+	+	+
54		+		+		+	+	+	+	+	+	+	+	+	+	+	+

^a Assay for complement dependent trypanolytic activity.

^b Sera obtained from trypanosome infected humans in the Lambwe Valley

^c WRATat number of the trypanosomes used as antigen in the lytic assay. W is Wellcome strain.

^d Indicates 100% lysis.

^e Indicates incomplete lysis.

developed in order to allow for a more complete determination of the antigenic types of trypanosomes experienced by humans in the Lambwe Valley.

C. Variable Antigen type (VAT) analysis of trypanosomes occurring in experimental Trypanosome rhodesiense infections.

Objective: To determine the VAT composition of the trypanosomes occurring by antigenic variation during the early stages of infection with cloned T. rhodesiense.

Description: The variant specific fluorescence assay previously described has led to the ability to detect and quantify antigenic variants at the level of the individual organism. This assay, together with the chronic nature of the Lambwe strain of Trypanosome rhodesiense in mice provides a useful system for the study of antigenic variation in African trypanosomiasis.

The work in this study deals with the occurrence of antigenic variants during the first two peaks of parasitemia in a chronic mouse infection with this strain of trypanosome. For this purpose, rabbit antisera against 13 different antigenic types of trypanosomes were used. As shown in the previous report, each of these antisera is specific for one of the WRATat's numbered 1 through 13. When used in an immunofluorescence assay, each antiserum reacts only with organisms of one variable antigenic type.

Progress: For the first part of this work, mice were infected with trypanosomes of WRATat 1 and subsequently assayed for the presence of trypanosomes of other antigenic types.

Figure 1 represents the parasitemia seen during the first two peaks of parasites in mouse No. 1, infected on Day 0 with 1×10^3 trypanosomes of antigenic type 1. A relapsing parasitemia can be seen, with the initial parasitemia peaking on day 6 of the infection. This peak is followed by a clearance of the parasites and subsequent relapse on day 13.

At the time that mice were bled from the tail for parasite counts, thin blood smears were also prepared. These smears were dried and stored at -20°C for later use in the immunofluorescence assay. At the time of assay, each blood smear was reacted with antisera to each of the 13 WRATats. For each antiserum, a total of 1000 trypanosomes were counted and the number giving positive fluorescence determined. This procedure allowed for a determination of the percentage of each antigen type present in the peripheral blood of the mice at the

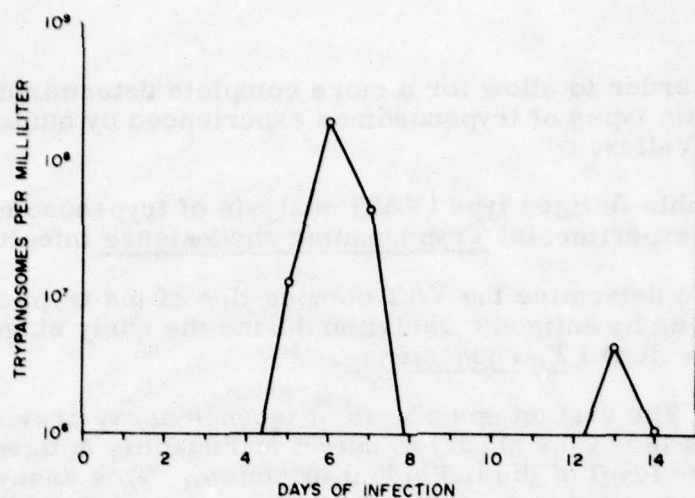


Fig. 1. Parasitemia in a mouse (#1) infected with WRATat 1.

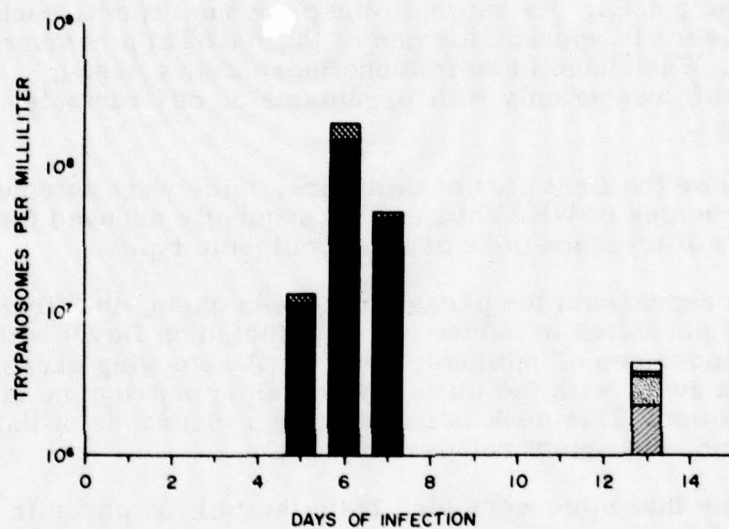


Fig. 2. Change in antigenic composition of trypanosome population during the course of infection with WRATat 1 (in mouse #1).

time the blood smears were taken.

The results using this assay for these two peaks of parasitemia in mouse No. 1 are shown in Figure 2. This figure shows the total parasitemia at each day of infection and also the antigenic composition of the trypanosomes present. The solid black areas of the bars represent the percentage of the organisms present found to be of the same antigenic type as the trypanosomes used to infect the animal, in this case WRATat 1. As can be seen, organisms of WRATat 1 comprised the majority of the trypanosomes present during the first peak. A second antigenic type was also found in the first peak, as shown by the crosshatched areas.

The second parasitemia, occurring a day 13 was less homogenous, as shown by the areas marked to represent three different antigenic types. There was also a fraction of the organisms which were not reactive with any of the 13 antisera available. These trypanosomes, represented by the open area of this bar were presumably of an antigenic type different from WRATat 1-13. Table 4 demonstrates the percentage of each WRATat present on day 6 of infection with WRATat 1 in five different mice. The percentage of each antigenic type detectable in these mice by the fluorescent assay is shown. WRATat's 2, 4-10 and 12 were not detected.

The composition of the starting inoculum was found to be 98% WRATat 1 and 1% WRATat 13 yielding a total percentage reactive of 99%. Mouse No. 1 shown in the previous figure is shown again here with 96% WRATat 1 and the remaining 4% WRATat 13 yielding a total of 100% reactive. WRATat 13, which was present in the starting inoculum, occurred in all five mice on day 6. Only two other antigenic types were detected. WRATat 3 occurred in mouse No. 2. These two mice also had a percentage of the organisms present which were not reactive with the available antisera.

The composition of the second peak of parasites in these same five mice is shown in Table 5. On day 12 of infection with WRATat 1, the indicated antigenic types were found. Types 1, 3, 6 and 7 were not detected. Also, some of the antigenic types occurred more frequently than others. For example, WRATat's 5 and 12 each occurred in 4 of the 5 mice while WRATat's 9, 10 and 11 each occurred in only one of the animals.

It can be seen from this table that the second peak of parasitemia was much more heterogenous than the first peak.

Table 4

Percentage of each WRATat present during the first peak of parasitemia

Day 6 of infection with WRATat 1

	WRATat Number	Calculated Total %	
		Reactive	Nonreactive
1	3	11	13
Inoculum	98	1	99
Mouse 1	96	4	100
Mouse 2	96	1	98
Mouse 3	78	13	92
Mouse 4	99	1	100
Mouse 5	99	1	100

WRATat 2, 4-10 and 12 were not detected

Table 5

Percentage of each WRATat present during the second peak of parasitemia

Day 12 of infection with WRATat 1

	2	4	5	8	9	10	11	12	13	Total % Reactive	Total % Nonreactive
Mouse 1	58	3						31		92	8
Mouse 2	4		14			1		4		23	77
Mouse 3			1			14		3	1	19	81
Mouse 4	4	6	2	1					19	32	68
Mouse 5			2	29	7			32		70	30

WRATat's 1, 3, 6 and were not detected

Also, a larger percentage of the trypanosomes present was non-reactive and thus presumably of antigenic types different from the 13 for which antisera were prepared.

The second part of this study deals with the occurrence of antigenic variants during the first two peaks of parasitemia after infection with WRATat 2. The same procedures described for the previous work were also used here.

Table 6 shows the percentage of each WRATat found on day 6 during infection with WRATat 2. Types 5-12 were not found. Once again it can be seen that in the first peak, the majority of the parasites were of the same antigenic type as the starting inoculum. Also, as before, there were some organisms that did not react with any of the antisera, both in the starting inoculum and on day 6 in four of the five mice.

Table 7 shows the antigenic type present in the second peak of parasitemia in these same mice. Again, many of the WRATat's occurred in these mice as indicated here with 7 different types present in the five mice, and no definite pattern of occurrence. Also a large percentage of the trypanosome were not reactive. It is interesting to note that WRATat 2 occurred in the second peak of parasitemia in mouse No. 2 even though this animal had shown clearance of WRATat 2 six days earlier. This apparent paradox remains unexplained.

Discussion: The occurrence of defined antigenic types of Trypanosoma rhodesiense in the first two peaks of parasitemia was investigated during experimental infection in mice. The first peak of parasites was found to be relatively homogeneous and predominantly of the same antigenic type as the starting inoculum. In contrast, the second peak of parasites was markedly heterogeneous, with as many as 5 different antigenic types occurring in the second peak in different mice infected with the same starting inoculum were greatly varied, i.e., no definite sequence was evident. However, some antigenic types were found more frequently than others.

In order to determine the practical implications of these results relative to the prospects for immunization, work is needed to determine the antigenic heterogeneity of the trypanosomes injected into the host by the fly vector and the heterogeneity of the first wave of parasites in the host.

II. Isolation and purification of protective antigens from Trypanosoma rhodesiense.

Table 6

Percentage of each WRATat present during the first peak of parasitemia
Day 6 of infection with WRATat 2

	WRATat Number					Calculated Total % Reactive	Calculated Total % Nonreactive
	1	2	3	4	13		
Inoculum	1	90			3	94	6
Mouse 1		79				79	21
Mouse 2		95				95	5
Mouse 3	16	73		1	16	106	0
Mouse 4		87	1			88	12
Mouse 5		98				98	2

WRATat's 5-12 were not detected

Table 7

Percentage of each WRATat present during the second peak of parasitemia
Day of 12 infection with WRATat 2

	WRATat Number					12	13	Calculated Total % Reactive	Calculated Total % Nonreactive
	2	4	5	6	11				
Mouse 1		1	1	1			7	10	10
Mouse 2	2				24			26	74
Mouse 3				1				1	99
Mouse 4			1			1	2	4	96
Mouse 5								7	93

WRATat's 1, 3, 7-10 were not detected

Objective: The purpose of this study was to continue work detailed in the previous annual report which showed that it was possible to obtain monospecific antiserum against a variant specific protein antigen.

Description: The previous report described antigenic components from two distinct variant specific trypanosomes obtained by DEAE-cellulose chromatography. Isoelectric focusing with wide range ampholines (pH 3.0-10.0) in thin layer acrylamide gels showed a number of major components distributed within the pH range 8-10. Cross-immunodiffusion of the isoelectric focused components with anti-trypanosomal sera resulted in precipitin lines which, when excised, washed and injected into rabbits, produced monospecific antisera. These findings were extended to include a comparative study of three different extract procedures. Briefly, the antigens compared were prepared as follows: 1) DEAE antigen is the breakthrough peak of freeze-thawed and syringe-disrupted trypanosomal extract; 2) KCl extract is prepared by extracting trypanosomal antigen with 3M KCl; and 3) Con-A represents the elution of absorbed soluble trypanosomal antigen to sepharose-concanavalin A column with methyl α -D-mannoside. This latter material was prepared by Dr. John Olenick and his associates in the Division of Biochemistry.

Progress: Figure 3 depicts the results obtained when three different extraction procedures of trypanosomal antigen, as well as different preparations of the same extraction procedure, are compared by isoelectric focusing in polyacrylamide gels.

When the isoelectric point of the bands are determined and compared with one another, several interesting observations can be made. First, although there are some differences among the three different preparation of W-3, it was remarkable how many bands are in common. Second, each of the DEAE preparations of W-3 possess cationic components that are either absent or present in extremely small amounts in the KCl and Con-A preparations. Third, every band identified in the KCl and/or Con A preparations can also be identified in the DEAE preparations. Fourth, there are several components in common in all W-3 preparations regardless of the extraction procedure.

The next experiment was designed to identify the specific band that represented the protective antigen. The three different preparations of W-3 were isoelectric focused in narrow range polyacrylamide gel (pH 7.5-10.0) in duplicate. One portion was fixed and stained to locate the position of the focused bands. The second, identical portion, was layered on antibody contain-

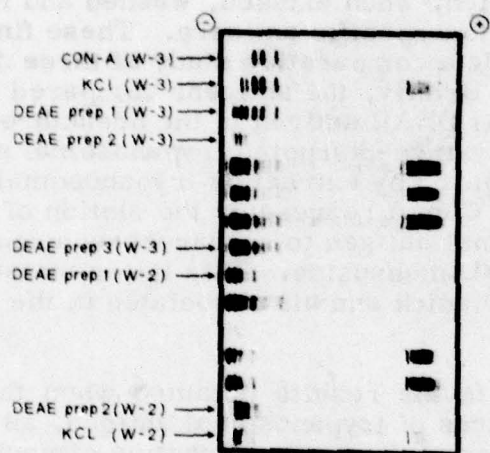


Fig. 3. Comparison of the various soluble antigen preparation of *T. rhodesiense* by isoelectric focusing in polyacrylamide gel (pH 3-10 range).

ing agarose. The results are depicted in Figure 4. It appears, that the size of precipitin rings are directly related to the intensity of the stained band; that is, the more protein the larger the precipitin ring size. It is very apparent, and disturbing, that all the separated bands are reacting with the monospecific antisera. However, it should be noted that the precipitin bands, particularly adjacent bands as demonstrated in DEAE, are not distinct but continuous which suggest identity between the various bands. These results could be explained on the basis of aggregation and/or fragmentation of the antigen during extraction or storage.

Molecular weight determinations of the three different W-3 preparations were done in polyacrylamide gradient gel electrophoresis in presence of 0.02% sodium dodecyl sulfate. Figure 5 illustrates the results obtained. The KCl extract was the most heterogeneous with material ranging from approximately 14,000 to 100,000 daltons; whereas, the Con A preparation exhibited the least heterogeneity. It is also apparent that the bulk of the protein, in all three different extraction procedures, was between 40,000 and 60,000 daltons.

Discussion: Continuation of the effort towards identification, isolation and characterization of the protective trypanosomal antigen. The major obstacle is the heterogeneity exhibited by the various preparations that most likely develops by aggregation and/or fragmentation of the antigenic moiety.

III. Complement Mediated Cytotoxicity Against Trypanosoma Rhodesiense Dependent on $F(Ab')_2$ of Specific IgG Antibody.

Objective: This project was designed to determine whether or not $F(Ab')_2$ derived from rabbit antibody against Trypanosoma rhodesiense is toxic to the organisms in the presence of complement.

Description: Previous studies in our laboratory have demonstrated that fresh serum from animals immunized against experimental African trypanosomiasis by the injection of gamma irradiated organisms is cytotoxic against the organisms as judged by inhibition of their capacity to incorporate radiolabelled leucine (J Immunol 116, 1005-1009, 1976). In these studies, heated immune serum in the absence of an added

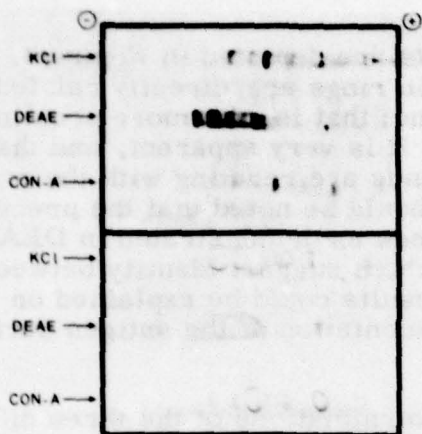


Fig. 4. Isoelectric focusing in polyacrylamide gel (pH 7.5 - 10.0 range) and antigen detection in situ by overlaying with antibody incorporated in agarose.

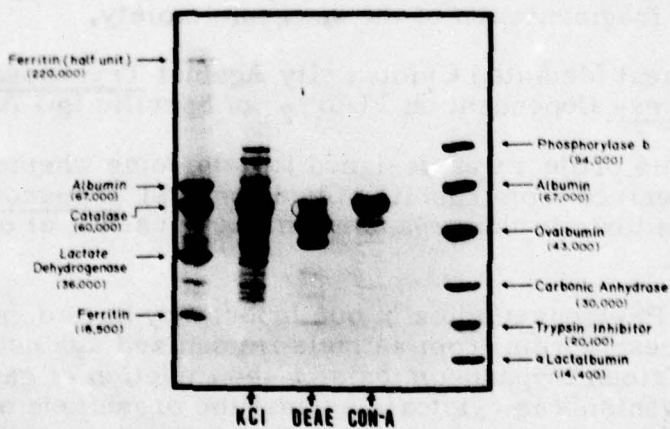


Fig. 5. Molecular weight determinations by polyacrylamide gradient gel electrophoresis in 0.2% sodium dodecyl sulfate (SDS).

source of complement did not sustain the cytotoxic reaction. Subsequently, we provided evidence that the complement requirement could be satisfied by the use of C4 deficient serum by the addition of EDTA but not EGTA, indicating that an alternative pathway supported the reaction (Infect. Immun., 19:928-93, 1978). We have also recently reported that depletion of factor B from human complement by affinity immunoabsorbent chromatography results in loss of the ability of the complement to support the cytotoxic reaction. The present study investigates the requirement for the Fc portion of rabbit IgG in this cytotoxic reaction. The immune rabbit serum was obtained by the immunization of animals with a soluble extract of the Wellcome strain of Trypanosoma rhodesiense using complete Freund's adjuvant.

Progress: IgG was isolated from immune and non-immune rabbit serum on staphylococcal protein A-sepharose columns. Portions of each of the preparations were then digested with pepsin at pH 4 for approximately 20 hours. Other portions of each preparation were incubated for the same period of time under identical conditions except for deletion of the enzyme from the mixtures. Following neutralization of the reaction mixtures they were again subjected to protein A-sepharose affinity chromatography. Figure 6 illustrates the patterns obtained with the immune IgG pepsin digest and its non-digested control. In the case of the control, a small amount of material did not bind to the column and is seen as a small peak of material absorbing at 280 nm; on eluting with acid buffer, the main peak of optical density was obtained and this was used for the experiments described. On the contrary, the major peak of absorbing material was obtained from the pepsin digest fractionation prior to application of the acid buffer; a small peak was obtained by acid elution, but this was discarded and the major peak used for the experiments.

The four protein preparations were used in in vitro cytotoxicity experiments: The organisms were incubated in the presence of the protein preparation and complement for 10 minutes, then, pulsed with tritiated leucine and incubated for an additional 60 minutes. The mixtures were then harvested on glass fiber filters, and the filter dried and counted in a liquid scintillation counter. It can be seen (Fig 7) that the immune IgG and $F(Ab')_2$ both markedly inhibited incorporation. Non-immune IgG and $F(Ab')_2$ led to essentially no inhibition at the lower concentration, although at higher concentrations, inhibition was seen. This inhibition of incorporation by non-immune IgG or serum has been seen consistently throughout our studies, and is believed to be due to interference with incorporation of the isotope and,

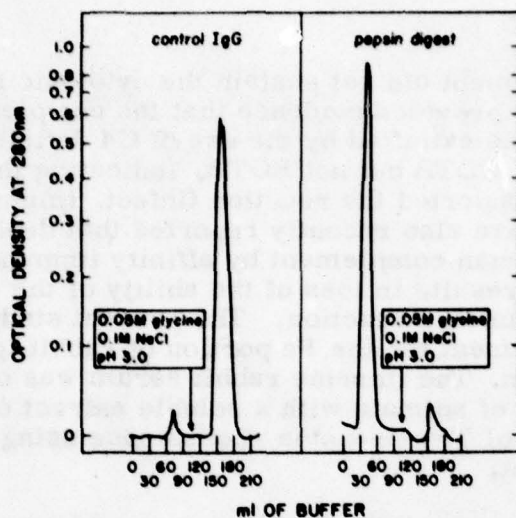


Fig. 6. Purification of anti-*Trypanosoma rhodesiense* IgG A1 F(Ab')₂ by staphylococcal protein A-sepharose affinity chromatography.

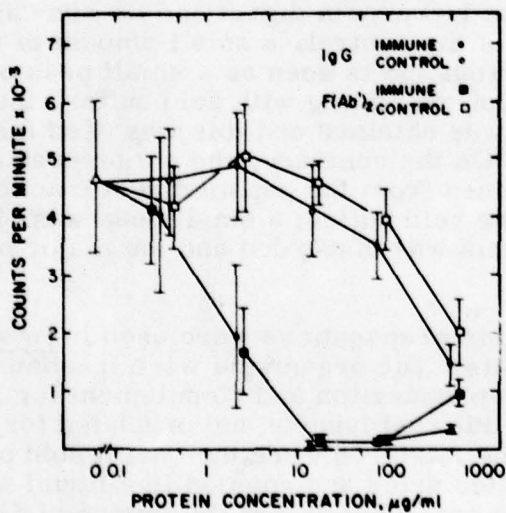


Fig. 7. Cytotoxicity against *Trypanosoma rhodesiense* by IgG and F(Ab')₂ antibody in the presence of complement

COMPLEMENT DEPENDENCE OF ANTIBODY INDUCED CYTOTOXICITY AGAINST

T. RHODESIENSE: EFFECT OF HEATING C₄ DEFICIENT SERUM

	EXPERIMENT #1	EXPERIMENT #2
	TREATMENT OF C4 DEFICIENT GUINEA PIG SERUM	MEAN % INHIBITION COMPARED TO NORMAL IgG
IMMUNOGLOBULIN (8 µg/ml)		CPM + 95% CONFIDENCE LIMITS
IMMUNE IgG	NONE	95 + 25
	30 Minutes	2544 + 213
NON-IMMUNE IgG	NONE	3058 + 507
	30 MINUTES AT 56°C	3077 + 1111
IMMUNE F(Ab') ₂	NONE	544 + 86
	30 MINUTES AT 56°C	2362 + 382
NON-IMMUNE F(Ab') ₂	NONE	2772 + 161
	30 MINUTES at 56°C	2249 + 732

*CPM = COUNTS PER MINUTE

Table 9
COMPLEMENT DEPENDENCE OF ANTIBODY INDUCED CYTOTOXICITY AGAINST
T. RHODESIENSE: EFFECT OF EDTA

IMMUNOGLOBULIN (8 µg/ml)	EXPERIMENT #1		EXPERIMENT #2	
	TREATMENT OF C4 DEFICIENT GUINEA PIG SERUM	MEAN & INHIBITION COMPARED TO NORMAL IgG	CPM* + 95% CONFIDENCE LIMITS	MEAN % INHIBITION COMPARED TO NORMAL IgG
IMMUNE IgG	NONE	96	278 + 42	97
	0.006 M EDTA	23	3742 + 1100	0
NON-IMMUNE IgG	NONE	-	7139 + 2540	-
	0.006 M EDTA	-	4808 + 2123	-
IMMUNE F (Ab') ₂	NONE	84	1070 + 403	81
	0.006 M EDTA	30	3944 + 965	0
NON-IMMUNE F (Ab') ₂	NONE	-	6410 + 1139	-
	0.006 M EDTA	-	5575 + 1056	-
			90 + 25	
			2559 + 351	
			3058 + 507	
			2536 + 885	
			544 + 86	
			2748 + 256	
			2772 + 161	
			2502 + 561	

* CPM = COUNTS PER MINUTE

Table 10

ANTIBODY INDUCED CYTOTOXICITY AGAINST T. RHODESIENSE IN THE

ABSENCE OF COMPLEMENT: EFFECT OF EDTA

IMMUNOGLOBULIN (8 µg/ml)	EDTA 0.006 M	EXPERIMENT # 1		EXPERIMENT # 2	
		MEAN % INHIBITION COMPARED TO NORMAL IgG	CPM* + 95% CONFIDENCE LIMITS	MEAN % INHIBITION COMPARED TO NORMAL IgG	CPM + 95% CONFIDENCE LIMITS
IMMUNE IgG	-	26	36350+ 10906	2	26284+ 10800
	+	15	49373 + 9139	1	30212+ 7189
NON-IMMUNE IgG	-	-	48692 + 5158	-	26767+ 2273
	+	-	57425+ 15663	-	30404+ 5936
IMMUNE F(ab') ₂	-	54	17359 1642	33	12294+ 1921
	+	33	28180+ 1534	34	14518 4430
NON-IMMUNE F(ab') ₂	-	-	37260+ 9142	-	18087 4076
	+	-	41900+ 4694	-	21889 2449

*CPM = COUNTS PER MINUTE

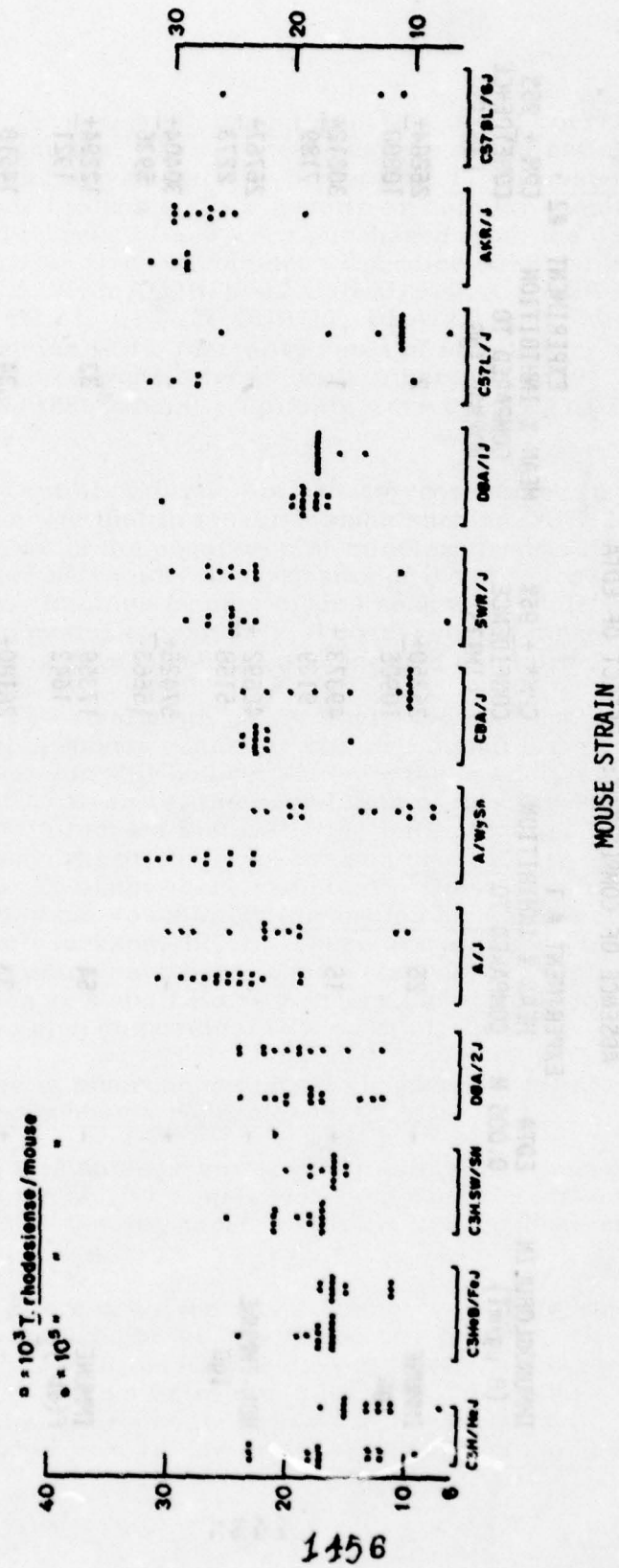


Fig. 8. Strain-dependent specificity of varied mouse strains to injections of 10^3 or 10^5 Trypanosoma rhodesiense.

based on microscopic observations, is not associated with cytotoxicity.

To further characterize the cytotoxic reaction by immune $F(Ab')_2$, studies were performed in which C4 deficient guinea pig serum was used as a source of complement. It can be seen (Table 8) that the material is also effective under these conditions. This inhibition of uptake can be reversed by heating the C4 deficient serum for thirty minutes at 56 C both in the case of intact IgG and in the case of $F(Ab')_2$ as indicated in both experiments.

Addition of EDTA to C4 deficient guinea pig serum also results in a reversal of the inhibition due to immune IgG or $F(Ab')_2$ (Table 9) this is evident in both experiments, although more obvious in the second.

Some inhibition of incorporation of the isotope in immune as compared to normal IgG or $F(Ab')_2$ can be seen in the absence of complement (Table 10) in the experiment reported the same concentration of immunoglobulins was used as in the previous two tables. It may be noted that the total counts obtained are much higher than in the presence of complement even though all other factors are comparable. On a percentage basis the inhibition obtained is considerably lower than that seen with complement and there is no consistent effect of EDTA on the extent of this inhibition.

Discussion and Recommendations: We conclude that the Fc portion of the IgG molecule is not required for the antibody dependent complement mediated killing of T. rhodesiense. Addition studies to further define the variations in effector mechanisms against this organism are required to fully describe the predictive value of in vitro assay as regards host immunity.

IV. Role of macrophages during the course of Trypanosoma rhodesiense infection in mice.

A. Strain-dependent specificity of mice to lethal effects of infection with Trypanosoma rhodesiense.

Objective: This investigation was designed to determine the resistance of various inbred mouse strains to infection with T. rhodesiense.

Description: Many inbred strains of mouse can be grouped into those susceptible or resistant to infection with certain bacteria, viruses or parasites. A prior report has shown that mortality

as a result of Trypanosoma brucei infection is not correlated with the H-2 complex. The present study is investigating whether the mortality of mice as a result of T. rhodesiense infection is related to some other genetic or cellular factors. Male mice 8-10 weeks of age were purchased from the Jackson Laboratories, Bar Harbor, Maine. The mice obtained were: A/J, AKR/J, A/WySn, C3H/HeJ, C3HeB/FeJ, C3H.SW/Sn, C57BL/6J, C57L/J, CBA/J, DBA/1J, DBA/2J, and SWR/J. In order to determine if the mortality rates of infected mice were dose-related, mice were injected with either 10^3 or 10^5 trypanosomes (EATRO 1886 strain). Animals were checked daily for death.

Progress: A partial collection of data is represented in Fig. 8. These data indicate that in mouse strains such as DBA/2J or C3H/HeJ, deaths in the population of mice exposed to 10^5 trypanosomes occur as rapidly as do deaths of those exposed to 10^3 trypanosomes. In other mouse strains such as C3HeB/FeJ and C3H.SW/Sn there did appear to be a dose-related correlation between numbers of trypanosomes injected and mortality.

Discussion: The strains which die early are not capable of the immunological response of the strains that do not die early. Further information will be derived from these studies after extensive genetic analyses at the completion of this investigation; however, it is of interest that C3H/HeJ mice were one of the strains most susceptible to Trypanosoma rhodesiense death, and C57BL/6J one of the more resistant. These results are consistent with those found in Trypanosoma brucei infections (Clayton, 1978; Clarkson, 1976). These are also the identical mouse strains which have been found to be respectively, LPS non-responders and LPS responders, as well as low-tumorcidal and high-tumorcidal (Ruco, et al. 1978).

B. Changes in macrophage cytostatic effect upon infection of mice with Trypanosoma rhodesiense.

Objective: This is an assessment of the effect of trypanosoma infection upon the ability of macrophage populations to inhibit proliferation of a specific target cell (i. e. macrophage activation).

Description: There are a number of tumor cell lines which will multiply (as measured by incorporation of a pulse of ^3H -labelled thymidine (^3H TdR) under proper conditions. When peritoneal macrophages from untreated mice are placed into culture with these tumor cells proliferation continues unabated. The addition of macrophages from mice chronically infected with

certain infections will inhibit growth (cytostasis) or kill the target cell population (cytotoxic effect). The present studies were designed to determine whether mice infected with T. rhodesiense developed a population of peritoneal macrophages which were cytostatic to the target cell RBL-5 (a murine Rauscher virus-induced cell line). C57BL/6J mice were injected with 10^3 T. rhodesiense or diluent. Ten days post-infection those mice previously injected with trypanosomes are given a booster injection of 10^7 or 10^6 gamma irradiated trypanosomes. Two to three days later the peritoneal macrophages of both control and experimental mice are harvested. Cells are plated at 4×10^5 cells/ml on plastic and permitted to adhere at 37 C in a 5%-CO₂ environment. After 60-90 minutes of incubation, plates are washed extensively of non-adherent cells, and placed upon ice. After 30 minutes of treatment cells are scrapped off the plates by means of a rubber spatula. Cells are then washed and plated at ratios of 10:1 to 80:1 (effector to target). Cultures are incubated approximately 44 hours with a 4 hour pulse of ³H thymidine prior to harvest. The RBL-5 target cells will continue to take up thymidine unless prevented by the presence of "activated" effector peritoneal macrophages.

Progress: A comparison of the incorporation of tritiated thymidine into RBL-5 target cells in the presence of macrophages from infected or untreated mice is presented in Table II. In these two representative samples, adherent cells from mice infected with trypanosomes permitted only 65% and 44% of the proliferation of RBL-5 as did cells from normal mice.

Discussion. This cytostatic assay has a number of inherent problems. Other investigators, as well as we, have found problems with consistency of the data from experiment to experiment. Some feel that the procedure of scraping adherent cells off plastic dishes results in damaged cells which behave inconsistently. It may also be that certain cell populations and cell types cannot interact reliably. To circumvent these problems we will simply add target tumor cells directly to macrophage monolayers harvested from untreated and infected mice. Also we will measure the cytotoxic, rather than the cytostatic potential of effector macrophages. The latter macrophage-target cell interaction, employing release of ⁵¹Chromium or tritiated thymidine results in more uniform, constant data.

Preliminary experiments in this laboratory to determine the cytotoxic potential of trypanosoma-infected mice indicated that the RBL-5 cell line may not be suitable for this type of assay. A different mouse cell line is currently under study.

Table 11

Incorporation of ^3H TdR by RBL-5 in the presence of macrophages from untreated and infected mice

	Cells	CPM	G. R. *
Exp. 1	Inf. PEC + media	616	.65
	Inf. PEC + RBL-5	75649	
Nor.	PEC + media	1733	
Nor.	PEC + RBL-5	116906	
Exp. 2	Inf. PEC + med	1588	.44
	Inf. PEC + RBL-5	7096	
	Nor. PEC + media	5267	
	Nor. PEC RBL-5	163330	
$*G. R. = \frac{(\text{CPM Inf PEC} + \text{RBL-5}) - (\text{CPM Inf PEC} + \text{media})}{\text{CPM Nor PEC} + \text{RBL-5} - (\text{CPM Nor PEC} + \text{media})}$			

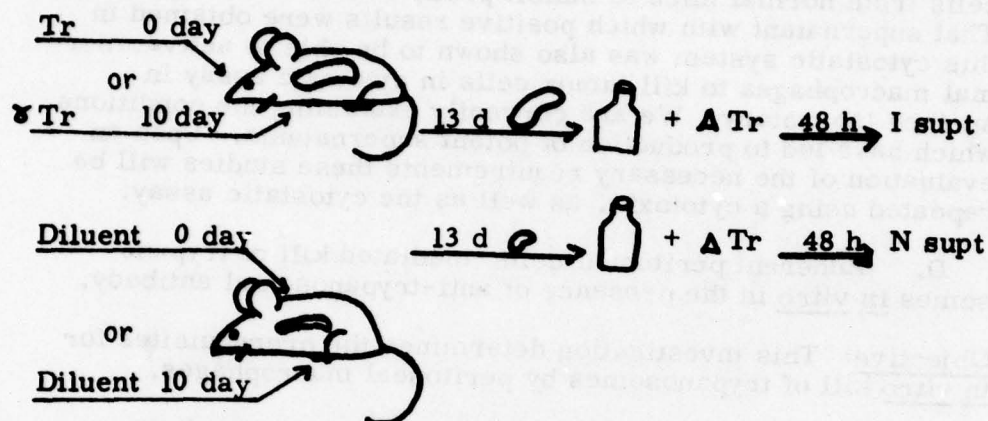
These results suggest that adherent cells from mice infected with trypanosomes have a heightened potential to inhibit cell proliferation of RBL-5.

C. Production of a splenic factor by infected mice which is capable of activating normal macrophages.

Objective. This study will determine whether spleen cell cultures from *Trypanosoma rhodesiense*-infected mice will produce factors upon incubation with trypanosome antigens that can activate normal macrophages to cytostatic or cytotoxic activity.

Description. Supernatants of PPD-stimulated BCG-immune spleen cell suspensions can activate normal peritoneal macrophages to kill tumor cells *in vitro* (Ruco and Meltzer, 1977). Since cells of mice infected with trypanosomes evidence capabilities of inhibiting tumor cell growth *in vitro*, it was likely that splenic cultures of these infected mice were producing supernatants which could activate peritoneal macrophages from untreated mice. Single cell suspensions of spleens from either untreated or trypanosome-infected mice were obtained. Cells were incubated in mass culture with trypanosome antigen (obtained by heating trypanosomes for two minutes at 56 C). Incubations were then carried out at 37 C in a 5% CO_2 -humidified

chamber. After 48 h stimulation, cells were pelleted by centrifugation and supernatants filtered through 0.22 μ m Millipore



filters. Peritoneal macrophages from untreated mice were incubated in 96-well microtiter plates at 37 C in a 5% CO environment. Sixty to ninety minutes later the wells were repeatedly washed by aspiration of non-adherent cells and replacement with media. Supernatants formed from antigen-stimulated splenic cultures were added to these macrophage monolayers for 4 hours prior to addition of RBL-5. Supernatants from trypanosome infected mice are expected to stimulate macrophages from untreated mice to inhibit proliferation of targets.

Progress: Supernatants, in one experiment, were upon addition to normal peritoneal mouse macrophages able to activate these cells to inhibit the normal proliferative rate of the tumor cell line (Table 12).

Table 12

Inhibition of incorporation of H TdR by RBL-5 in the presence of untreated macrophages and splenic factors

Culture	CPM	G. R.
N PEC + media + RBL-5	229299	-
N PEC + I supt* + RBL-5	128324	.56
N PEC + N supt* + RBL-5	231411	1.00

*I supt and N = supernatant obtained, respectively, from "immune" and untreated murine splenic cell suspensions incubated with trypanosomal antigen for 48 h.

Discussion: The spleen cells of mice infected with trypanosomes produce a factor which may activate peritoneal adherent cells from normal mice to inhibit proliferation of a target cell. That supernatant with which positive results were obtained in this cytostatic system was also shown to be able to activate normal macrophages to kill tumor cells in cytotoxic assay in another laboratory. We are currently examining the conditions which have led to production of potent supernatants. Upon an evaluation of the necessary requirements these studies will be repeated using a cytotoxic, as well as the cytostatic assay.

D. Adherent peritoneal cells mediated kill of trypanosomes in vitro in the presence of anti-trypanosomal antibody.

Objective: This investigation determines the prerequisites for in vitro kill of trypanosomes by peritoneal macrophages.

Description. Documentation from these laboratories have shown that serum from animals infected with Trypanosoma rhodesiense can, in the presence of complement, kill trypanosomes in vitro (as measured by the decrease of incorporation of ^3H leucine as an assay of trypanosome death). To examine whether macrophages can substitute for the absolute requirement of complement in this system the following procedure was carried out. Peritoneal macrophages from untreated mice were incubated for 2 hours at 37 C in a CO_2 incubator before washing to remove nonadherent cell populations. Wellcome strain trypanosomes were then placed on these monolayers. Either immediately thereafter, or 2 hour post-incubation at 37 C, an aliquot of trypanosomes were removed, diluted, and injected into ICR mice. Any trypanosome damaged or phagocytized by the macrophage monolayers would not be able to multiply and kill the host.

Progress and Discussion: Studies indicate that neither normal macrophages nor macrophages from trypanosome-infected mice are capable of killing trypanosomes by themselves. Microscopic examination of monolayer-trypanosome combinations after as many as 24 hour incubation show no changes in trypanosomal mobility. A comparison of the incorporation of radiolabelled leucine by trypanosomes in the presence and absence of various populations of macrophages is under study. Studies are also planned to determine the influence of IgG and F(ab)_2 fragments from T. rhodesiense infected mouse sera upon macrophage handling of live trypanosomes.

V. Evaluation of Immunofluorescence in the Diagnoses of Early Cutaneous Leishmaniasis

Objective: The objective of this study was to evaluate indirect immunofluorescence as a method of the diagnosis of early cutaneous leishmaniasis contracted by troops on training in the Canal Zone.

Description: These studies were performed using sera obtained during the course of a project conducted by Ft. Bragg of troops observed before and after potential exposure to leishmaniasis during training at the jungle warfare training center in the Canal Zone. Sera were obtained just prior to departure and after return from the endemic area approximately 2 months after termination of the period of exposure. A fluorescent antibody test was developed based on that previously described by Walton et al. with certain modifications. The antigen consisted of Leishmania braziliensis (Panama) amastigotes from axenic culture stored in 20% glycerol at -72 C. Slides were prepared and the organisms fixed on the day of the test. The unknown serum was diluted 1/16 and applied to the antigen slide for 20 minutes after which the slide was washed 6 times. Goat anti-human globulins at a 1/150 dilution in 0.1% Evans blue was applied to preparations after which they were incubated for 15 minutes and then washed 6 times. The unknown sera in the study were examined for fluorescence by two observers using the recommendations regarding surface fluorescence as described by Walton.

Conditions differed somewhat from those described by Walton in that amastigotes derived from Vero cell cultures were used in his paper and material of this type was not available at the time the current studies were performed. Other variables which differed in the two procedures were studied systematically and no differences found among them; these included the use of frozen slides versus bulk frozen antigen, the use of Evans blue at 0.2%, 0.1%, or completely omitted; unfixed versus 2% formalin fixed antigen preparations; the use of veronal versus phosphate buffer, and the use or omission of bovine serum albumin in the diluent. Table 13 illustrates the criteria used for interpretation for interpretation of the fluorescence patterns observed.

Progress: Table 14 demonstrates an antiglobulin titration using a normal and a positive control serum. It can be seen that the positive control, which was taken from a long standing culture positive case of cutaneous leishmaniasis reacts strongly in this experiment. A number of other sera were tested demonstrating a high degree of false positives among sera from patients with

Table 13

Cutaneous Leishmaniasis IFAT
Interpretation of fluorescence pattern

Pattern	Grade	Interpretation
all organisms fluoresce (2 observers)	2-4+	Reactive
50% or fewer organisms fluoresce (2 observers)	-, \pm , or 1+	Non reactive
two observers disagree		Weakly reactive

Table 14

Cutaneous leishmaniasis IFAT
antiglobulin titrations

Serum	Dilution	Antiglobulin Dilution					
		1/4	1/50	1/80	1/100	1/120	1/200*
Normal	1/8	+	-	-	-	-	-
Positive Control	1/8	4+	3+	3+	3+	2-3+	2+
Normal	1/16	-	-	-	-	-	-
Positive Control	1/16	3+	3+	3+	2+	2-3+	2+

*With Evans blue, 0.1%

various parasitic diseases (Table 15). Since it was assumed that the troops would be free of parasitic diseases prior to exposure, assays were performed on all of the 610 sera. It can be seen in Table 16 that during the course these assays at approximately 30% of the putatively normal sera (ie., sera from laboratory personnel at the WRAIR with no history of protozoan disease) gave false positive reactions. Over all, 65% of the non-leishmaniasis sera were positive during these assays. The results were not available until after the end of the series since they were all coded and read blind by the primary and secondary readers. Table 17 summarizes the result of sera from the airborne troops. It can be seen that the number of reactive sera is almost identical pre and post exposure. Similarly, the negative to positive conversion rate was almost identical to the positive results with the results of culture (Table 18) it is clear that seroconversion was unrelated to acquisition of disease in this series. Only a single individual of the 10 who became culture positive exhibited sero conversion. The results presented above were confirmed by an independent assay of all 10 culture positive patients and a sample of the other sera from Ft. Sherman by Dr. Walton's laboratory in the Canal Zone.

Discussion and Recommendations: It is clear from these results that the indirect fluorescent antibody test for cutaneous leishmaniasis as currently performed is not useful for the diagnosis of the early disease. The control sera used for setting up the test with cases of long standing disease clearly represents another category of patients with respect to the assay. A further difficulty with the test is the appearance of a high proportion of false positives under the conditions employed, both among individuals with other parasitic diseases and among individuals who have no known infection. There is a serious need for a laboratory test for the detection of early leishmaniasis which would, ideally, precede the appearance of lesions, and would, in any case, not require physical examination. Further work towards this goal is recommended.

VI. Mechanisms of Cellular Immunity to Plasmodium berghei Sporozoites.

Objective: The objective of this series of investigations is the application of in vitro techniques to elucidate possible mechanisms of cell mediated immunity to malaria sporozoites using the Plasmodium berghei-BALB/c mouse model.

Description: Sporozoites, the transmissible form of the malaria parasite nature, have been attenuated and successfully used as

Table 15

Cutaneous leishmaniasis IFAT
reactivity of various sera

Serum Category	Positive/total tested
Normal	0/4
Chagas"	2/2
African trypanosomiasis	1/1
Schistosomiasis	1/1 9/15+60% "false positives"
Malaria	
falciparum	3/4
malaria	1/1
vivax	1/2
Cutaneous leishmaniasis (culture positive)	2/3

Table 16

Cutaneous leishmaniasis IFAT reactivity of blind controls
during assay of Ft. Bragg Sera (14 Assays)*

Serum category	Positive/total tested	
Normal	11/34	
Malaria	21/22	46/71 = 65%
Trypanosomiasis	12/13	"false positives"
Helminthiasis	2/2	
Cutaneous leishmaniasis	14/16	88% sensitivity

*one observer

Table 17

Cutaneous leishmaniasis IFAT pre and post Ft. Sherman
exposure of 610 troops

Number of reactive sera:	Pre exposure	128	(21%)
	Post exposure	134	(22%)
Conversion rates:	Non reactive to reactive	76	(12%)
	Reactive to non reactive	70	(11%)
	Remained non reactive	406	(67%)
	Remained reactive	58	(10%)
	Total	610	

Table 18

Cutaneous Leishmaniasis IFAT
Sensitivity and specificity by criterion of culture

Serologic Conversion	Culture		total	
	+	-		
+	1 (10%)*	75 (13%)	76	(12%)
-	9 (90%)	525 (87%)**	534	(88%)
total	10	600	610	

*Sensitivity = 10%

**Specificity = 87%

immunogens in the protection of man, higher primates, and rodents against subsequent sporozoite induced malaria infection. Recent studies at this and other institutes have demonstrated that ant sporozoite immunity is T-cell dependent. Effector mechanisms in lymphocyte-parasite interaction are not known. Three possible mechanisms are being studied.

1. The direct cytolysis of sporozoites by specifically sensitized lymphocytes is investigated by the direct exposure in vitro of sporozoites to splenocytes which have been isolated from immune mice. After incubation with lymphocytes, the sporozoites are inoculated into mice to determine their infectivity. The protocol for this experiment is being validated by comparison to ^{51}Cr -labelled mastocytoma target cell lysis by sensitized splenocytes under identical conditions.

2. The possibility for direct neutralization of sporozoites by lymphokines produced by sensitized lymphocytes is being investigated by exposure of sporozoites in vitro to supernatants from cultures of sensitized lymphocytes which have been recovered from immune mice. The sporozoites are subsequently inoculated into mice to determine their infectivity.

3. The mediation of phagocyte activity by sensitized lymphocyte is being studied in vivo. Mice are immunized against P. berghei sporozoites, depleted of their phagocytic cells by treatment with silica, and challenged to determine if phagocytic cells play a direct role in effecting immunity to sporozoites.

Progress: Preliminary experiments have demonstrated the feasibility of maintaining freshly isolated P. berghei sporozoites in vitro at 37 C in Leibovitz-L-15 medium with 10% heat inactivated fetal calf serum for a period of one hour without a substantial loss of their infectivity. The same medium-FCS system can readily support the viability of mouse splenocytes at 37 C in normal atmosphere for periods exceeding 72 hours. These observations have allowed study of CMI to sporozoites in vitro to proceed. A series of five experiments were conducted to determine if immune BALB/c mice would diminish sporozoite infectivity. Immune or normal plates at a concentration of 2×10^5 cells per ml of medium at 37 C for 24, 48, or 72 hours. In order to control for the physical changes in the aliquots of medium without cells were incubated in microtiter plates concurrently with the cell cultures and in this report will be referred to as aged medium. After incubation supernatants were removed from cell cultures and sporozoites were suspended in supernatants from sensitized or normal

cells or aged medium at a concentration of 2×10^4 sporozoites per ml of supernatant and incubated for 60 minutes at 37 in a water bath. After incubation, sporozoites were resuspended and inoculated into female BALB/c mice, 1×10^4 sporozoites per mouse. Mice were bled on alternate days beginning 3 days post-inoculation and the percentage of mice successfully infected was recorded. The results were compared with a control group of mice receiving immediated inoculation of sporozoites which had not been exposed to either cell culture supernatants or aged medium.

Results of these experiments are presented in Table 19. As the data indicate, the results of Experiment 1 would seem to indicate that supernatant from sensitized cell cultures has a deleterious affect upon sporozoite the data indicates the results obtained in experiment 1 would seem to indicate infectivity; however, these results were not corroborated by further experimentation. Within any given experiment there is little difference between the infectivity of sporozoites incubated with supernatant from sensitized cells, normal cells, or aged medium. Comparison of the infectivity of incubated sporozoites with the infectivity of sporozoites that were inoculated immediately demonstrates that there is some loss of infectivity due to maintenance of the sporozoites in vitro, but this loss does not appear to be significant in most cases.

Another series of experiments was performed to determine if direct contract with sensitized splenocytes would have any effect upon the infectivity of sporozoites. Sporozoites were incubated for one hour at 37 C with either sensitized splenocytes or normals splenocytes at a cell to sporozoite ratio of 50 to 1. After the incubation period the sporozoites and splenocytes were resuspended and inoculated into mice. Since it was not possible to separate the splenocytes from the sporozoites before inoculation, each mouse received 5×10^5 sensitized or normal splenocytes along with 10^4 sporozoites. In order to control for the possibility of passive transfer of immunity by the splenocytes, other groups of mice were inoculated with sporozoites and sensitized splenocytes or sporozoites and normal splenocytes that were mixed together and inoculated immediately without pre-incubation. Another control group included mice which received sporozoites only without incubation. Table 20 shows the percentage of mice infected in each group. In the first two experiments performed there is little difference in groups of sporozoites exposed in vitro to sensitized splenocytes and normal splenocytes. Again it can be noted that incubation in vitro did decrease the infectivity of sporozoites somewhat as compared to sporozoites inoculated immediately. Additional replicates of these experiments are presently being conducted.

Table 19
Effects of splenocyte culture supernatants upon sporozoite infectivity
Percent Mice Infected
with Sporozoites Incubated with

Exp No.	Cell Culture Time (hrs.)	Supernatant from Sensitized Cells	Supernatant from Normal Cells	Aged Medium+	Immediate Inoculation*
1	24	43	100	80	
2	72	90	100	100	100
3	72	91	92	88	75
4	24	56	11	56	100
5	48	100	100	100	100
Mean	—	76	81	85	95

+medium inoculated for designated culture time without cells

*sporozoites isolated at 4 degrees and not incubated before inoculation

Table 20
Effects of incubation with splenocytes upon sporozoite infectivity

Exp No.	% Mice Infected by Sporozoites Incubated with		% Mice Infected After Inoculation with	
	Sensitized Splenocytes	Normal Splenocytes	Sporozoites* +Sensitized Splenocytes	Sporozoites Normal +Splenocytes Only
1	50	57	100	86
2	75	66	—	—
Mean	60	62	—	—

*sporozoites and cells mixed and inoculated immediately into mice.

In preparation for experiments to determine the role of phagocytic cells in the immunity to sporozoites, a protocol for depletion of macrophage by silica inoculation has been established. Briefly the protocol is as follows: Mice are given 25 mgs of colloidal silica iv 48 hours prior to immunization or challenge by sporozoites. Twenty-four hours prior to immunization or challenge the mice are given an additional dose of 3 mg of silica iv. Mice which have received this treatment demonstrate a significant reduction in their ability to clear colloidal carbon particles from their circulation as compared to mice receiving no silica treatment and mice receiving either IP inoculation of silica or iv inoculation of silica only. This protocol will be used to study the effects of phagocytic cell depletion upon immunity to sporozoites.

Discussion: The demonstration of the importance of specifically sensitized T-lymphocytes in ant sporozoite immunity by Spitalny, et al. (1977), Chen, et al. (1977), and Hockmeyer, et al. (1978) has left open the question as to the exact role of these cells in protection against sporozoites. Several roles of T-cells in immunity to various microorganisms are known. These functions include acting as helper cells in the production of antibody by B-cells, the production of lymphokines which are directly cytotoxic for microorganisms or activate phagocytic cells to destroy microorganisms, and the direct cytolysis of microorganisms by T-cells themselves. Although immune animals produce antibody to sporozoites, the demonstration of ant sporozoite immunity in the absence of detectable antibody (Spitalny and Nussenzweig, 1972) would seem to indicate that helper activity is neither the only nor the primary role of T-cells in sporozoite immunity.

Papernmaster, et al. (1976) demonstrated the *in vivo* regression of tumors as a result of local administration of supernatants from cultures of immune cells, and Kanra and Vesikari (1975) have shown cytotoxic activity against rubella infected cells in the supernatants of rubella virus stimulated human lymphocyte cultures. Present experiments have failed to demonstrate the production by immune splenocytes of any soluble factor that is cytotoxic for sporozoites under the experimental conditions used. Experiments conducted so far have likewise failed to demonstrate a direct cytotoxic activity of immune splenocytes against sporozoites. Since only two experiments have been conducted, further attempts will be made to demonstrate a direct cytotoxic effect. Experimental conditions for these experiments will first be established using immune splenocyte effector cells and ⁵¹CR-labeled mouse mastocytoma cells as targets. Identical conditions will be used to

study the immune splenocyte-sporozoite cytotoxicity system. If immune splenocytes are found to be cytotoxic for sporozoites, experiments will be repeated using T-cell enriched lymphocyte populations as effector cells.

Macrophages can participate in both the afferent limbs of the immune response to sporozoites. Macrophages can be involved in processing antigen for presentation to T-cells or can be activated by sensitized T-cells to phagocytize sporozoites. Primary interest at this point is in determining whether or not macrophages are active in effecting the immune response to sporozoites. Preliminary experiments will determine whether or not sensitized animals retain their immunity to sporozoite challenge after their phagocytic cell populations have been depleted by treatment with silica. Definitive experiments will determine if the presence of phagocytic cells in recipient animals is a prerequisite of successful passive transfer of immunity to sporozoites.

Project 3M762770A803 Malaria Prophylaxis

Work Unit 083 Protective immunity in protozoan diseases

Literature Cited.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a		2. DATE OF SUMMARY ^a		3. REPORT CONTROL SYMBOL	
77 10 01 D. Change U U				DA OB 6495		78 10 01		DD-DR&E(AR)836	
4. SUMMARY SCTY ^a 5. WORK SECURITY ^a				7. REGRADING ^a NA		8. DESIG'S INSTN ^a NL		9. SPECIFIC DATA - CONTRACTOR ACCESS ^a <input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO. / CODES ^a PROGRAM ELEMENT PROJECT NUMBER				TASK AREA NUMBER		WORK UNIT NUMBER		11. LEVEL OF SUM A. WORK UNIT	
A. PRIMARY 62770A 3M162770A803				00		084			
B. CONTRIBUTING									
C. KNOWLEDGE CARDS 114F									
12. TITLE (precede with Security Classification Code) ^a									
(U) Synthesis of Antiparasitic Drugs									
13. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a									
012100 Organic Chemistry									
14. START DATE 72 07			15. ESTIMATED COMPLETION DATE CONT			16. FUNDING AGENCY DA		17. PERFORMANCE METHOD C. In-House	
18. CONTRACT/GRANT			19. DATES/EFFECTIVE: NA			20. RESOURCES ESTIMATE		21. PROFESSIONAL MAN YRS	
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D. KIND OF AWARD			E. CUM. AMT.			CURRENT 79		5.0	
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NAME ^a Walter Reed Army Institute of Research			NAME ^a Walter Reed Army Institute of Research			25. FISCAL YEAR		433	
ADDRESS ^a Washington, DC 20012			ADDRESS ^a Division of Experimental Therapeutics Washington, DC 20012			26. PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. A. contract institution)			
RESPONSIBLE INDIVIDUAL			NAME ^a Sweeney, T.R., Ph.D.			TELEPHONE: 202/576-3731		SOCIAL SECURITY ACCOUNT NUMBER	
NAME: RAPMUND, G., COL			ASSOCIATE INVESTIGATORS			NAME: Canfield, C.J., COL		NAME	
TELEPHONE: 202/576-3551									
27. GENERAL USE									
Foreign intelligence not considered									
28. REVIEW (Precede EACH with Security Classification Code)									
(U) Malaria; (U) Leishmaniasis; (U) Trypanosomiasis; (U) Schistosomiasis; (U) Antiparasitic Drugs; (U) Chemical Synthesis; (U) Antimalarials									
29. TECHNICAL OBJECTIVE ^a 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)									
23. (U) The objective is to manage, integrate, and provide technical direction for both a contract and in-house program to obtain potentially active antiparasitic agents for military use through rational organic syntheses.									
24. (U) Necessary research areas are defined, proposed research evaluated, ongoing research guided, evaluated, and integrated with the other program elements. Technical advice is obtained through an Ad Hoc Study Group on Medicinal Chemistry. Information is exchanged by contractors through technical meetings.									
25. (U) 77 10-78 09 Programs for the synthesis of potentially useful antileishmanial and antitrypanosomal agents have been initiated. The synthetic effort for antileishmanial agents involves 8-aminolepidines and that for antitrypanosomal agents centers on nucleosides. The main effort in the synthesis of antimalarial agents is in the area of 8-aminolepidines which show curative activity against both blood- and sporozoite-induced infections. Two series of acridine derivatives have also shown excellent activity and are being vigorously pursued. A series of thiosemicarbazones have been made; some of these show very good antimalarial activity and wide spectrum <i>in vitro</i> antibacterial activity. The development of a modified computerized chemical and biological data handling system has received major attention and should be operational by the end of CY 78. During the year approximately 170 target compounds were synthesized in the program and submitted for testing. Three compounds were synthesized in large quantities in the preparations laboratories for advanced testing and preclinical studies. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.									

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

1477

Project 3M162770A803 MALARIA PROPHYLAXIS

Work Unit 084 Synthesis of Antiparasitic Drugs

Investigators:

Principal: Thomas R. Sweeney, Ph.D.

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Bing T. Poon, Ph.D.; Daniel L. Klayman, Ph.D.;
CPT John P. Scovill, Ph.D.; Edgar A. Steck,
Ph.D.; Richard E. Strube, Ph.D.

The Research Contract Chemical Synthesis Program

During this reporting period there were 13 contractual synthesis programs devoted to the design and synthesis of new antimalarial agents. Continuing research areas that are based upon biochemical rationales include the synthesis of potential blood schizontocidal compounds designed to inhibit the enzyme hypoxanthine phosphoribosyltransferase, thymidylate synthetase and dihydrofolate reductase. Other research efforts are based upon improving the efficacy and reducing the toxicity of 8-aminoquinolines and 8-aminolepidines as potential tissue schizontocidal agents. Finally, synthetic development of leads derived from patent disclosures is also in progress.

The four highly active classes of compounds that were reported last year as emerging from the synthesis program, *viz.*, 5-aryloxy-6-methoxy-8-aminolepidines, new Mannich bases related to amodiaquine, acridine diones and imine derivatives of acridine diones, have been vigorously pursued. One 8-aminolepidine has been selected for IND preparation, toxicity studies are being carried out on three Mannich bases to make a final selection of one for an IND preparation and the synthesis efforts in the other two areas are being phased out because a selection of a representative from these classes for an IND preparation can be made as soon as all biological testing is finished and results compiled. It is anticipated that patent applications will be filed on all four of these classes of drugs.

One area of research was dropped during the year, *viz.*, the synthesis of compounds designed to interfere with parasite phospholipid synthesis because of the inordinate basic chemical research effort required to arrive at desired compounds.

A modest effort, i.e., two contracts, is continuing in the synthesis of potential antileishmanial agents. This effort is confined to 8-aminolepidines; it is in this class that the most active antileishmanial compounds to date have been uncovered.

Three new contracts were let during the year. Two of these are concerned with the synthesis of nucleosides as potential antitrypanosomal or antischistosomal agents; specifically the target compounds are based upon modification of tubercidin and nucleocidin both of which were reported to have antiparasitic activity. Finally, a very small effort is being devoted to the synthesis of substituted benzocarbazoles, a class of compounds possessing antitrypanosomal activity.

The Preparations Laboratories

The two preparations laboratories are used chiefly to re-synthesize large quantities of selected compounds that are needed for clinical, pharmacological, or large animal studies. On occasion they may make a large quantity of an intermediate that would be generally useful in the synthesis program.

The output of the preparations laboratories is summarized in the following table. It should be recognized that some of the compounds received may have been requested in an earlier reporting period and some requested in this period will not be received until the next period.

FY - 77-78	Target Compounds				Total
	Quantities (g)			Intermediates	
	>1000	100-1000	<100		
Number requested	5	16	28	10	59
Number received	3	13	18	16	50

Data Processing

1. Inventory

The new chemical inventory system is fully operational and functioning. Draft documentation has been prepared and delivered. The shipping cycle is run every third working day and periodic large reports generated according to schedule.

2. Chemistry

The new chemistry programs have been tested on a test base and appear to be functional. The data base is about 70% converted, with completion scheduled for Spring of CY-1979. Search programs and integrated chemistry-inventory reports will be tested on the incomplete data base early in FY-79. Output from these tests can be used to check the status of the data base, test the programs, and be used for decisions on shipping samples for biologic screening.

Documentation for the chemistry system is being drafted and should be completed by mid FY-79. On line editing and correction of structures for the data base is currently being done both inhouse and contractually.

All volatile noxious chemical samples were specially sealed to reduce odors in the area where chemicals are stored and handled.

3. Biology

Conversion programs for biology data processing are nearly complete, and the integration of biology processing inhouse with chemistry and inventory is scheduled for the 1st quarter of FY-79.

Acquisition of Compounds

The following table summarizes the number of various classes of compounds received during FY-78.

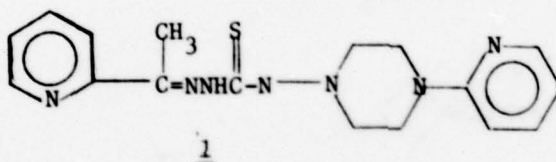
	<u>Originals</u>	<u>Duplicates</u>	<u>Total</u>
Purchased	123	18	141
Gifts	61	29	90
Synthesized	421	183	604
Discreet	3090	813	3903
Prep labs	22	41	63
Open combinations	5	0	5
Discreet combinations	4	0	4
Total	<u>3726</u>	<u>1084</u>	<u>4810</u>

Twelve companies submitted compounds under the no-dollar agreement during the reporting period. Five new agreements were signed and two are under negotiation.

Organic Synthesis Laboratory

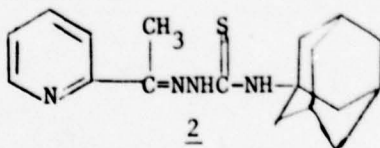
During the past year, the most striking development has been the discovery that many of the 2-acetylpyridine thiosemicarbazones which have been synthesized for their potential antimalarial properties also have strong inhibitory activity against certain bacteria. Many new structures have been designed, therefore, with both antimalarial and specific antibacterial activities in mind.

Over 50 new thiosemicarbazones have been submitted for antimalarial screening over the past year, the best of which is compound 10. The latter has



shown cures in mice infected with Plasmodium berghei at a dose as low as 20 mg/kg.

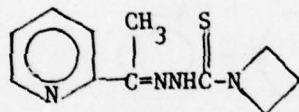
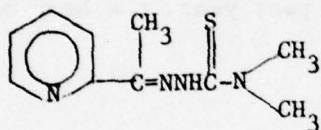
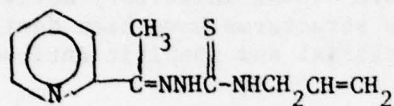
At WRAMC, Dr. Dobek has noted fair antibacterial activity in a select group of 2-acetylpyridine thiosemicarbazones against the following bacteria: Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella dysenteriae, E. coli, and Proteus vulgaris. Higher activity was seen in Group D Enterococcus where compound 2 had an MIC (minimum inhibitory concentration) of 1 $\mu\text{g/ml}$



and in Staph. aureus where group of 2-acetylpyridine thiosemicarbazone including 2 had MIC's in the range of 0.5 to 1 $\mu\text{g/ml}$.

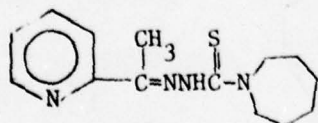
A higher level of in vitro activity of 2-acetylpyridine thiosemicarbazones was found against penicillin-sensitive and resistant strains of Neisseria gonorrhoeae (MIC's) as low as <0.015 $\mu\text{g/ml}$). In N. meningitidis MIC's were observed in the range 0.031-0.125 $\mu\text{g/ml}$ for two derivatives.

The effect of our thiosemicarbazones is being studied on Mycobacterium smegmatis at Johns Hopkins University inasmuch as the action of an inhibitory agent is believed to be prognosticative of inhibition against M. leprae. The following three compounds show MIC's of <1 to 3 $\mu\text{g/ml}$:



The 2-acetylpyridine thiosemicarbazones are not cross-resistant with the antileprosy drugs, Rifampin, Clofazimine, DDS or Thiacetazone. It is anticipated that the leprosy mouse foot-pad test will be run at C.D.C., Atlanta, on 2 or 3 of the active compounds in early 1979.

In *M. intracellulare*, responsible for an atypical tuberculosis, the most potent compound (MIC = 0.1 $\mu\text{g/ml}$) found in tests conducted at the National Jewish Hospital and Research Center, Denver, is:



An animal model is being refined so that the activity of this compound can be further explored.

A series of 4-amino-2-methoxy-9-methylacridines is being synthesized. The amino group is being substituted with so-called antimalarial side chains. The initial compounds are undergoing testing at present.

Studies on the purification of antiradiation agent, WR 2721, have led to an improved recrystallization procedure which gives the compound as a stable trihydrate. The purity of the suitably treated WR 2721 appears to be superior to what has been seen heretofore.

The isolation and determination of primaquine, its analogs and metabolites from biological systems has been studied extensively using a gas chromatographic technique.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^b	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY ^c	4. KIND OF SUMMARY ^d	5. SUMMARY SCTY ^e	6. WORK SECURITY ^f	7. REGRADING ^g	8a. DISSEM INSTN ^h	8b. SPECIFIC DATA - CONTRACTOR ACCESS ⁱ	9. LEVEL OF R&D ^j
77 10 01	D. Change	U	U	NA	NI	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
10. NO./CODES ^k	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER	
a. PRIMARY	62770A	3M162770A803		00		086	
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						
11. TITLE (Precede with Security Classification Code) ^l							
(U) Biological Evaluation of Antimalarial drugs							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^m							
012600 Pharmacology 002600 Biology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
66 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT NA				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
a. DATE/EFFECTIVE:				PREVIOUS		78	
b. NUMBER:				FISCAL		3.8	
c. TYPE:				YEAR		236	
d. KIND OF AWARD:				CURRENT		3.9	
e. CUM. AMT.				79		206	
20. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, DC 20012				Div of Experimental Therapeutics Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
NAME: RAPMUND, COL G.				NAME: Davidson, D. E., LTC			
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-2292			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS			
				NAME:			
				NAME:			
22. KEYWORDS (Precede EACH with Security Classification Code)							
(U) Plasmodium; (U) Malaria; (U) Drug Development; (U) Antimalarials; (U) Biology; (U) Chemistry; (U) Pharmacodynamics; (U) Drug Metabolism; (U) Toxicology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To conduct in-house and contract studies in biology specifically related to the design, development and exploitation of new antimalarials for military use against drug resistant strains.							
24. (U) Close supervision will be maintained by providing guidance and an integrated evaluation of productivity, and by the redirection and coordination of objectives as dictated by feedback from clinical studies as candidate antimalarials.							
25. (U) 77 10 - 78 09. Approximately 9000 compounds were screened for suppressive, causal prophylactic, or radical curative antimalarial activity in animal models in-house and at 6 contractor laboratories. Approximately 500 had activity. Of these, approximately 100 have been selected for advanced study, including 15 which are being tested against human malarials in Aotus monkeys. A culture system for evaluation of candidate drugs against drug sensitive and drug resistant P. falciparum in vitro has been established. Activity of standard drugs indicates that results of this test are highly predictive of activity in man. A new class of 8-aminoquinolines with 6 times the tissue schizonticidal activity of primaquine and with potent blood schizonticidal activity are undergoing preclinical investigation. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77-30 Sep 78.							

DD FORM 1498

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68, FOR ARMY USE, ARE OBSOLETE.

1484

Project 3M162770A803 MALARIA PROPHYLAXIS

Work Unit 086 Biological Evaluation of Antimalarial Drugs

Investigators

Principal: LTC David E. Davidson, Jr., VC

Associate: MAJ Robert E. Desjardins, MC; Gerald J. McCormick, Ph.D.; Gloria P. Willet

1. Description

Screening and testing of candidate antimalarial compounds by Dept of Parasitology, WRAIR, and by contractor laboratories under technical direction of Dept of Parasitology has continued at a somewhat reduced rate compared to previous years.

2. Progress

a. Blood Schizonticidal Testing:

The search for new blood schizonticidal drugs to be used for prophylaxis and treatment of drug resistant falciparum malaria in man continues. Primary screening in the mouse/Plasmodium berghei blood schizonticidal test (University of Miami) is being conducted at a rate of 120 compounds per week. In FY 78, approximately 6000 compounds were tested. Nearly 500 compounds exhibited activity.

Of the 500 compounds active in the primary mouse screen, approximately 200 have been selected for further testing in vitro, in advanced rodent tests and in primate malaria models. Nine compounds have exhibited sufficient promise of potential clinical utility to have been selected for pre-clinical testing in the Aotus monkey/ Plasmodium falciparum model, and testing has been completed on 9. Schizonticidal testing in the rhesus monkey/Plasmodium cynomolgi model has been suspended due to non-availability of test animals.

b. Tissue Schizonticidal Testing

The search for better causal prophylactic and radical curative antimalarial drugs continues. Primary screening is performed at a rate of 45 compounds per week in the mouse model utilizing sporozoite-induced P. berghei yoelli malaria.

Secondary mouse testing in the causal prophylactic model of Peters, et al. is used to resolve ambiguities in activity created in the primary screen by distinguishing between true causal prophylactic activity and the blood schizonticidal activity of persistent compounds. Agreement between the mouse causal prophylactic test systems and the rhesus monkey radical curative test has been approximately 80%.

The rhesus monkey radical curative test which utilizes sporozoite-induced Plasmodium cynomolgi malaria is considered to be the model most closely resembling vivax malaria in man. During this year 59 compounds have been tested in this model in a screening mode, and of these 41 exhibited activity sufficiently greater than primaquine to warrant more quantitative follow-up study in the rhesus model. One compound, WR 225448, is undergoing intensive preclinical efficacy trials in the rhesus model.

c. Development of an In vitro Antimalarial Drug Screen

1). Background

During the past year, a new in vitro system for evaluating the antimalarial potency of candidate drugs against both chloroquine-sensitive and chloroquine-resistant strains of Plasmodium falciparum has been developed in this laboratory. The technique is based on an automated microtiter system using standard 96-well microtiter plates.

The parasites are maintained in vitro by a modification of a continuous culture method previously described (Haynes, Diggs, Desjardins and Hines, Nature 263:767-769, October 1976, additional manuscript in preparation).

2). Methods

An initial volume of 25 μ l of culture medium is added to each well of the microtiter plate (manually or with a semiautomated device - Cooke Laboratory Products, Microdrop I System). Each compound is dissolved initially in sterile pyrogen-free water or 100% ethanol and diluted to 70% ethanol. This solution is allowed to stand at room temperature for 15-20 minutes to kill potential bacterial contaminants in the culture. An aliquot of this solution is then further diluted in culture medium containing 10% human plasma. The initial concentration of the compound is calculated based on its expected approximate

potency against the strain of P. falciparum to be evaluated. Manually 25 μ l of the medium containing compound is added to duplicate wells in Row B of the microtiter plate. An automatic diluter (Cooke Laboratory Products) is then used to make serial 2-fold dilutions from Row B through Row H of the microtiter plate. This results in a 64-fold range of concentrations with duplicate columns for each compound. Finally, 200 μ l of culture medium with ~1.5% RBC (type A or type O) with 0.5-1.0% parasitemia is added to each well except wells A9-A12. To these wells a similar suspension of parasite-free RBC's is added. Row A (wells 1-8) then serves as drug-free parasite control wells, and Row A (wells 9-12) serves as drug-free RBC control wells. After addition of the 200 μ l of RBC's in medium, the highest concentration of ethanol is less than 0.1%--a concentration we have demonstrated to have no effect on parasite growth and replication.

The plates are then preincubated for 24 hours in a reduced oxygen enhanced carbon dioxide atmosphere at 37°C as described in the forementioned publication. Finally 25 μ l of culture medium containing 20 μ Ci/ml of 3 H-hypoxanthine is added to each well and the plate incubated as above for an additional 18 hours.

After the 18 hour isotope pulse the 96 well plates are harvested on an automated harvester (MASH II - Microbiologic Associates), each well being washed copiously with distilled water. This device deposits the particulate contents of each well on a 3 mm fiberglass disk which is in turn placed in a scintillation vial with 5.0 ml of Hydromix^R for counting (Searle - Model Delta 300 Scintillation Counter).

3). Data Analysis

A teletype punch tape records the data for subsequent reading and analysis by a table-top programmable calculator (Tektronix Model 4051). The duplicate data for each compound are then fit by nonlinear regression techniques to a logistic-logarithmic regression model to generate an estimate of the ED-50 with 95% confidence limits, i.e.,

$$Y = \frac{(U - L)}{2} [1 + \tan h (\beta \log C - \beta \log X)] + L$$

Where Y = DPM, X = concentration, U = upper limit of function, L = lower limit of function, β = slope of the tangent function, and C = ED-50.

To date, approximately 20 different compounds have been evaluated against four different strains of P. falciparum. The known antimalarials, chloroquine, mefloquine and quinine, have been repeatedly tested against the Uganda I (chloroquine-sensitive) and Smith (chloroquine-, quinine-, and pyrimethamine-resistant) strains of P. falciparum.

4). Results

Tables 1-3 show results obtained with a number of antimalarial compounds tested against the Uganda I and Smith strains. Sample graphs generated by the Tektronix 4051 Graphics Calculator System are also included in Figures 1-6. Two sulfonamides were ineffective, presumably because of the presence of abundant PABA and folic acid in the medium we are now using.

We have also demonstrated the ability of the system to detect and quantify the antimalarial activity in the plasma of a patient treated with chloroquine though this data is also being further evaluated.

5). Plans

We intend to develop this system further along the following lines:

1). Optimization of initial parasite load in the 36 hour incubation and 18 hour pulsing period.

2). Alteration of the medium (reduction of PABA and folic acid content) to extend the range of utility to include other potentially useful antimalarials.

3). Determination of the pharmacokinetics of parent compounds by chemical analysis and parallel measurements of plasma-associated antimalarial activity for drugs in current stages of clinical and preclinical development using human volunteers and laboratory animals. This will establish the identity or non-identity of the parent compound as the active antimalarial in vivo.

4). Use of in vitro human liver microsome preparations (obtained from transplant donors) to evaluate potentially useful metabolites of known and potential antimalarials.

5). Extension of present results with other strains of P. falciparum with known sensitivities and resistance (e.g., Ethiopian Tamenie, Cambodian Buchanan and possibly Vietnam Marks).

6). Incorporation of a second tracer (probably ^{14}C -amino acid) in the system adapted to dual label counting. This will involve very little additional technical work and will potentially extend the range of utility in detecting anti-parasitic activity (specifically with regard to mechanism of drug action).

7). Finally, with the automation of the system, it is proposed that this could serve as a potential primary screen for new candidate antimalarials yielding information on potential activity against both sensitive and resistant strains of the parasite.

6). Discussion

The feasibility, potential output and utility of this system to the Antimalarial Drug Development Program are clear. The ability of the system to detect nanogram quantities of active antimalarial drugs in human plasma and the potential for use of in vitro microsome-generated metabolites greatly extend the potential of the system beyond that of a simple in vitro assay. The highly quantitative and reproducible nature of the data generated are also significant. In addition, as a research tool, the culture system offers many opportunities to study the biochemistry of the parasite in relation to various pharmacologic manipulations. Considerable effort should, therefore, be directed toward further development of this in vitro antimalarial assay system.

Table 1: Suppression of Incorporation of ^3H -Hypoxanthine in Cultures of Two Strains of *P. falciparum* by Anti-malarial Quinolines

Drug	Suppression of Uptake of ^3H -Hypoxanthine:	
	ED ₅₀ (ng/ml) \pm S.D.*	
Chloroquine	9.5 \pm 0.78 (n = 12)	182.0 \pm 23.37 (n = 12)
Quinine	36.1 \pm 5.57 (n = 9)	108.6 \pm 8.45 (n = 9)
Amodiaquine	9.8 \pm 0.45 (n = 2)	23.7 \pm 5.15 (n = 2)
Mefloquine	6.7 \pm 1.00 (n = 12)	7.8 \pm 1.54 (n = 12)

* Mean \pm standard deviation of the mean; n = number of replications

Table 2: Suppression of Incorporation of ^3H -Hypoxanthine in Cultures of Two strains of P. falciparum by Sulfadiazine and Two Antifolates.

<u>Drug</u>	Suppression of Uptake of ^3H -Hypoxanthine: <u>ED₅₀ (ng/mg) \pm S.D.*</u>	
	<u>African Uganda I</u>	<u>Vietnam Smith</u>
Pyrimethamine	5.1 (n = 1)	1500 (n = 1)
WR 158,122**	52.0 \pm 16.7 (n = 2)	>138 (n = 2)
Sulfadiazine	>138 (n = 1)	>138 (n = 1)

* Mean \pm standard deviation of the mean; n = number of replications.

** WR 158,122: A 2,4-diamino-6-substituted-quinazoline.

Table 3: Suppression of Incorporation of ^3H -Hypoxanthine in Cultures of Two Strains of P. falciparum by Candidate Antimalarial Drugs

Drug **	Suppression of Uptake of ^3H -Hypoxanthine: ED ₅₀ (ng/ml) \pm S.D.*	
	<u>African Uganda 1</u>	<u>Vietnam Smith</u>
Primaquine	>138	>138
WR 184,806	10.0	7.7
WR 180,409	35.4	48.5
WR 194,965	4.6	6.5
WR 172,435	7.9	3.2

* Mean \pm standard deviation of the mean; n = number of replications

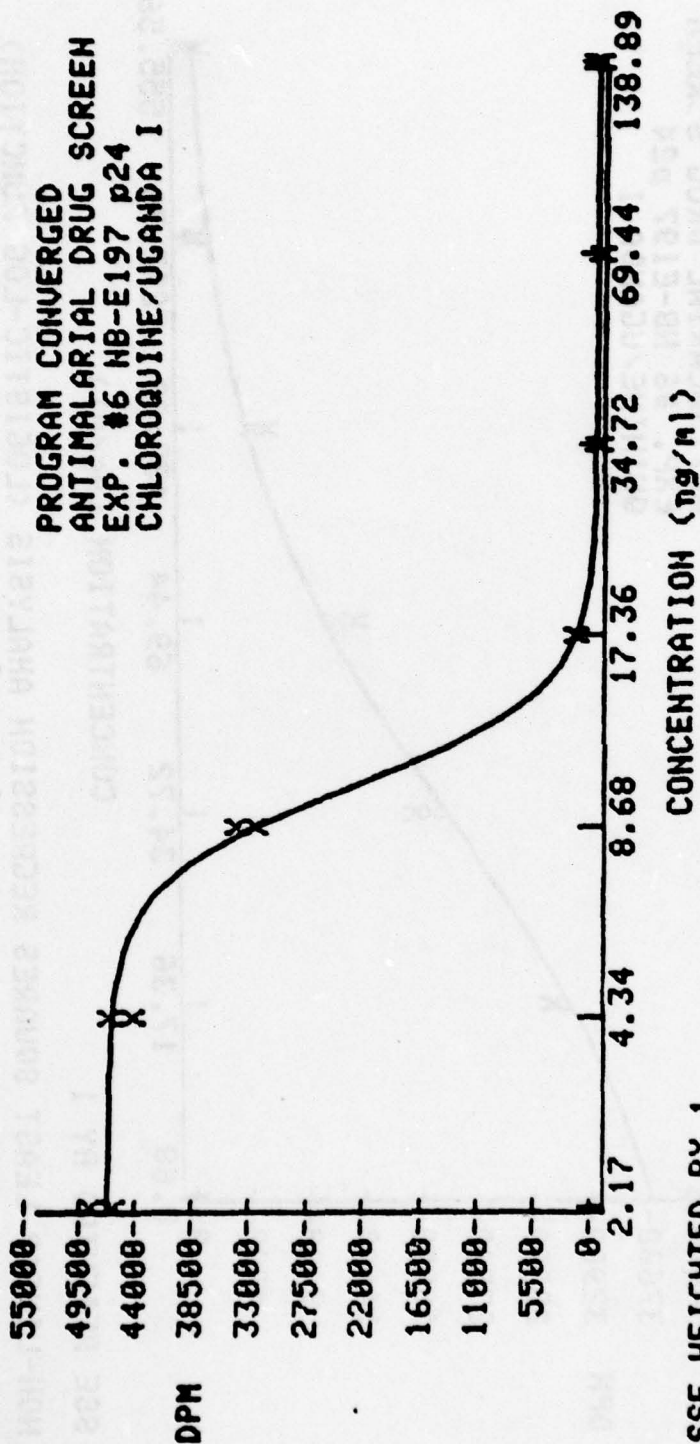
** WR 184,806: A 4-quinolinemethanol

WR 180,409: A 2,6-disubstituted-4-pyridinemethanol

WR 194,965: An o-cresol

WR 172,435: A 2,6-disubstituted-4-pyridinemethanol

Figure 1: Suppression of ^3H -Hypoxanthine Incorporation in Uganda I Strain of P. falciparum by Chloroquine



SSE WEIGHTED BY 1

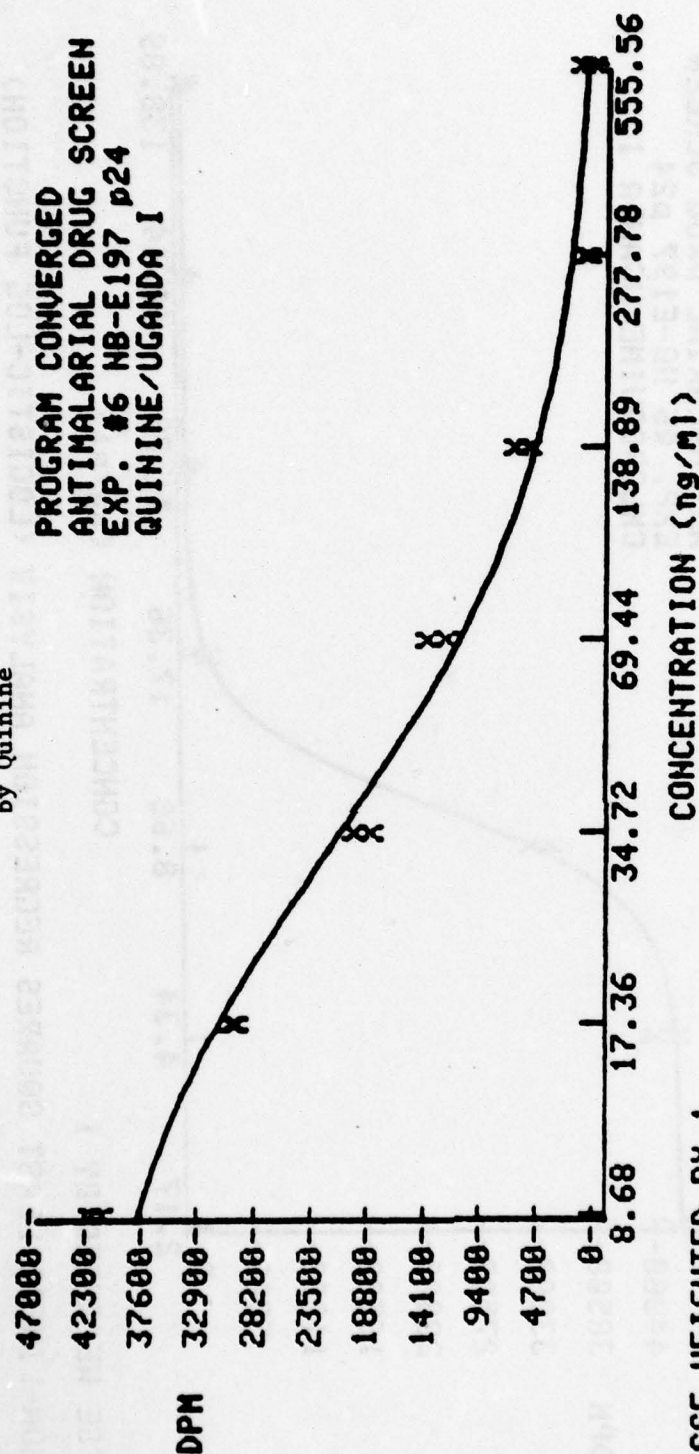
NON-LINEAR LEAST SQUARES REGRESSION ANALYSIS (LOGISTIC-LOG FUNCTION)

^3H -HYPOXANTHINE LABEL (UPTAKE) vs. DRUG CONCENTRATION

ED-50 = 10.2492 ng/ml

95% C.I. = (9.9300 TO 10.5682) (R^2 = 0.9983) 19 OCT. 1977

Figure 2: Suppression of ^3H -Hypoxanthine Incorporation in Uganda I Strain P. falciparum by Quinine



SSE WEIGHTED BY 1

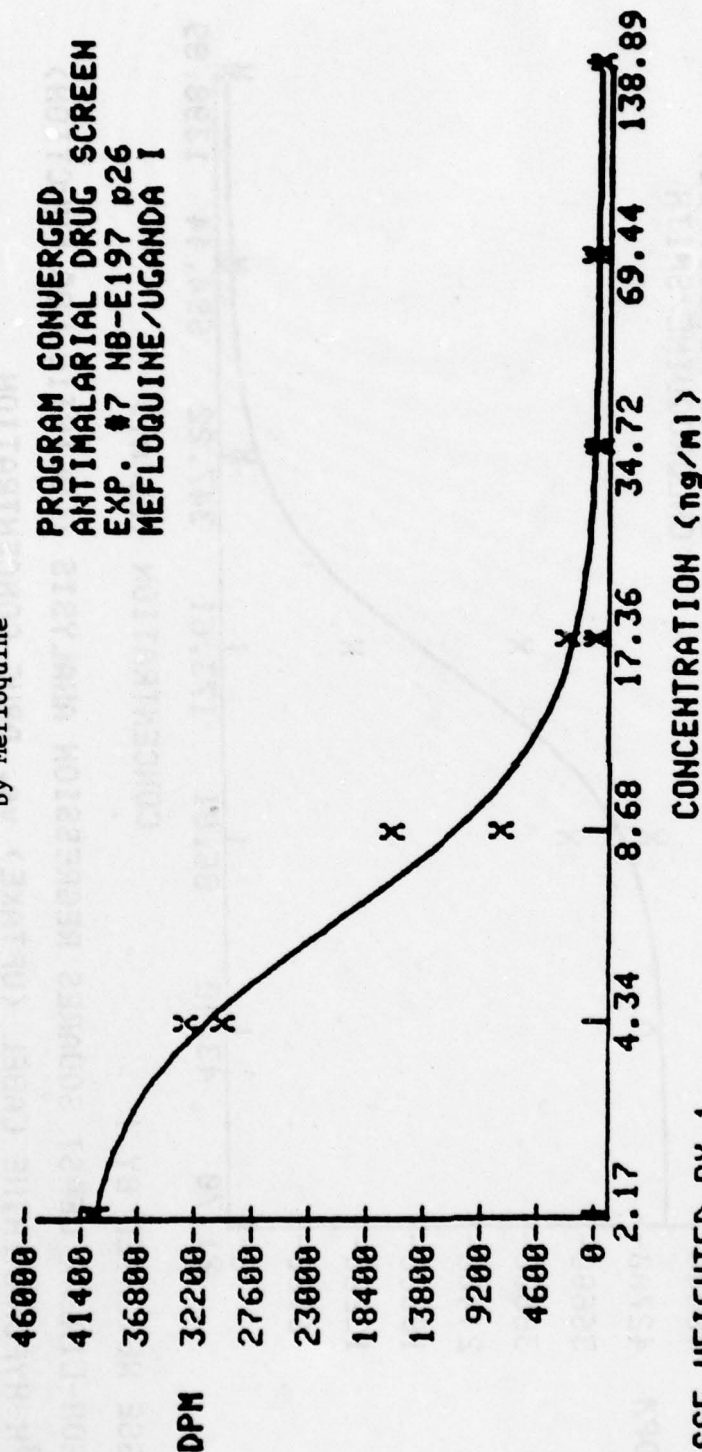
NON-LINEAR LEAST SQUARES REGRESSION ANALYSIS (LOGISTIC-LOG FUNCTION)

^3H -HYPOXANTHINE LABEL (UPTAKE) vs. DRUG CONCENTRATION

ED-50 = 34.5129 ng/ml

95% C.I. = (30.5362 TO 38.4889) (R² = 0.9814) E197

Figure 3: Suppression of ³H-Hypoxanthine Incorporation in Uganda I Strain P. Falciparum by Mefloquine



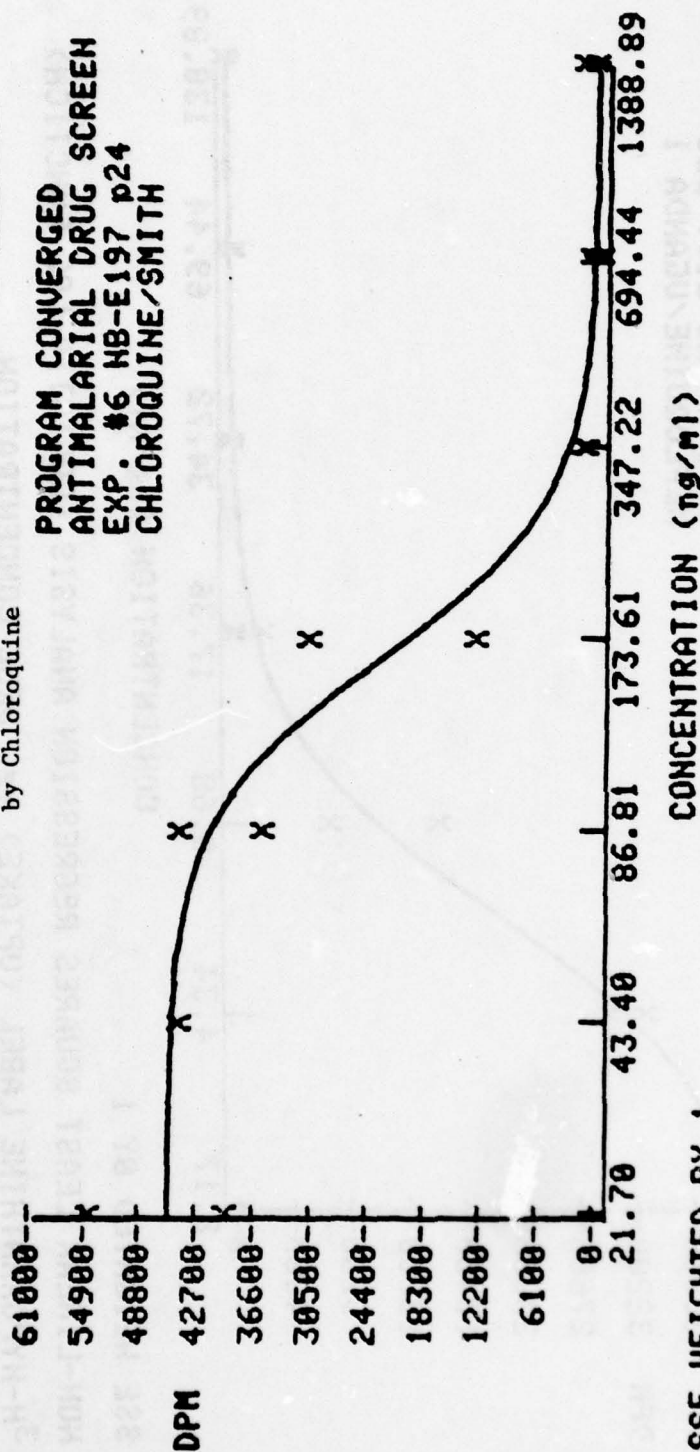
NON-LINEAR LEAST SQUARES REGRESSION ANALYSIS (LOGISTIC-LOG FUNCTION)

³H-HYPOXANTHINE LABEL (UPTAKE) vs. DRUG CONCENTRATION

ED-50 = 6.3857 ng/ml

95% C.I. = (5.8851 TO 6.8862) (R² = 0.9865) 26 OCT. 1977

Figure 4: Suppression of ^3H -Hypoxanthine Incorporation in Smith Strain P. falciparum
by Chloroquine



SSE WEIGHTED BY 1

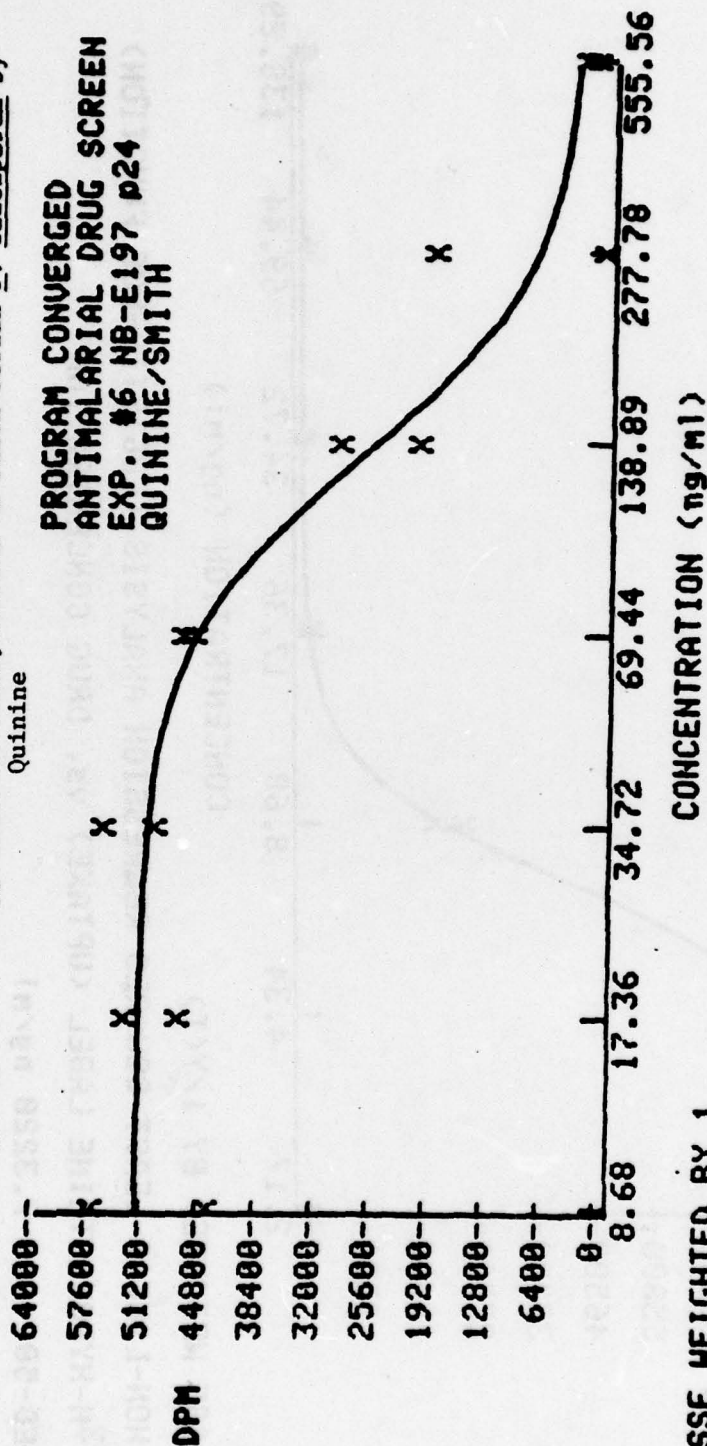
NON-LINEAR LEAST SQUARES REGRESSION ANALYSIS (LOGISTIC-LOG FUNCTION)

^3H -HYPOXANTHINE LABEL (UPTAKE) vs. DRUG CONCENTRATION

ED-50 = 166.3642 ng/ml

95% C.I. = (138.8104 TO 193.9146) (R^2 = 0.9452) 19 OCT. 1977

Figure 5: Suppression of ^3H -Hypoxanthine Incorporation in Smith Strain P. falciparum by Quinine



NON-LINEAR LEAST SQUARES REGRESSION ANALYSIS (LOGISTIC-LOG FUNCTION)

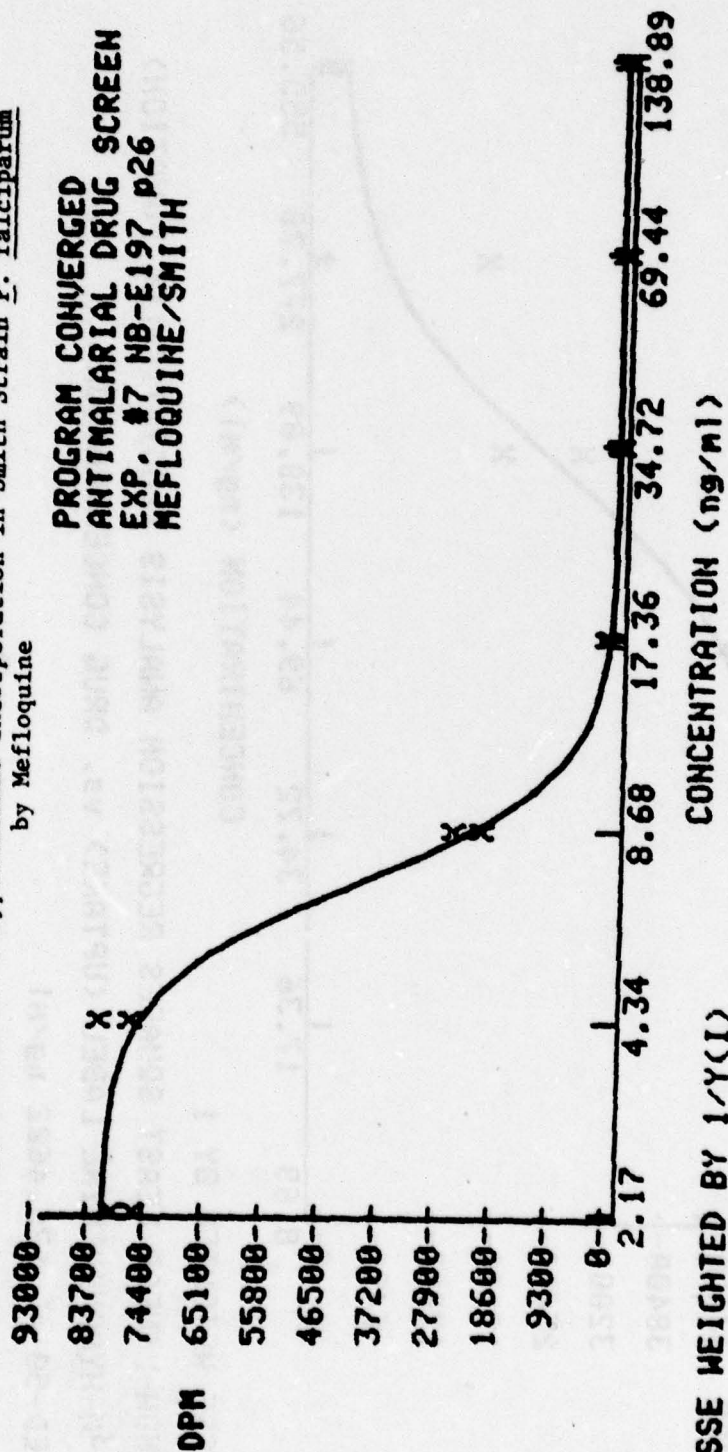
^3H -HYPOXANTHINE LABEL (UPTAKE) vs. DRUG CONCENTRATION

ED-50 = 136.4602 ng/ml

95% C.I. = (111.1847 TO 161.7329) (R^2 = 0.9357) 19 OCT. 1977

Figure 6: Suppression of ³H-Hypoxanthine Incorporation in Smith Strain P. falciparum
by Mefloquine

PROGRAM CONVERGED
ANTIMALARIAL DRUG SCREEN
EXP. #7 NB-E197 p26
MEFLOQUINE/SMITH



SSE WEIGHTED BY 1/Y(I)

NON-LINEAR LEAST SQUARES REGRESSION ANALYSIS (LOGISTIC-LOG FUNCTION)

³H-HYPOXANTHINE LABEL (UPTAKE) vs. DRUG CONCENTRATION

ED-50 = 7.3220 ng/ml

95% C.I. = (6.9353 TO 7.7084) (R² = 0.9927) 26 OCT. 1977

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION		2. DATE OF SUMMARY		REPORT CONTROL SYMBOL	
				DA OB 6536		78 10 01		DD-DR&E(AR)636	
3. DATE PREV SUMMARY		4. KIND OF SUMMARY		5. SUMMARY SCTY		6. WORK SECURITY		7. REGRADING	
77 10 01		D. Change		U		U		NA	
8. NO. / CODES		9. PROGRAM ELEMENT		10. PROJECT NUMBER		11. TASK AREA NUMBER		12. WORK UNIT NUMBER	
		62770A		3M 62770A803		00		087	
13. PRIMARY		14. CONTRIBUTING		15. CONTRIBUTING		16. CARDS 114F			
17. TITLE (Precede with Security Classification Code)									
(U) Determination of Pharmacological Effects of Antimalarial Drugs									
18. SCIENTIFIC AND TECHNOLOGICAL AREA									
012600 Pharmacology 002600 Biology									
19. START DATE		20. ESTIMATED COMPLETION DATE		21. FUNDING AGENCY		22. PERFORMANCE METHOD			
72 07		CONT		DA		C. In-house			
23. CONTRACT/GRANT				24. RESOURCES ESTIMATE		25. PROFESSIONAL MAN YRS		26. FUNDS (In thousands)	
a. DATES/EFFECTIVE: NA				b. PREESTIMATE		c. 9.0		d. 365	
e. NUMBER: NA				f. FISCAL YEAR		g. 78		h. 79	
i. TYPE:				j. AMOUNT:		k. 9.0		l. 390	
m. KIND OF AWARD:				n. CUM. AMT:					
27. RESPONSIBLE DOD ORGANIZATION				28. PERFORMING ORGANIZATION					
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research					
ADDRESS: Washington, DC 20012				ADDRESS: Washington, DC 20012					
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Anomalous Institution)					
NAME: RAPMUND, COL G.				NAME: HEIFFER, Dr. M. H.					
TELEPHONE: 202-576-3551				TELEPHONE: 202-576-3014					
29. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:					
Foreign intelligence not considered				ASSOCIATE INVESTIGATORS					
				NAME: CHUNG, Dr. H.					
				NAME: ROZMAN, Dr. R. S.					
29. KEYWORDS (Precede each with Security Classification Code) (U) Pharmacodynamics; (U) Pharmacokinetics; (U) Toxicology; (U) Antimalarial Drugs; (U) Preclinical Pharmacology; (U) Human Volunteer									
30. TECHNICAL OBJECTIVE, 31. APPROACH, 32. PROGRESS (Furnish individual paragraphs identified by number precede text of each with Security Classification Code.)									
<p>23. (U) The technical objectives are to develop and exploit animal models for the study of the pharmacodynamic and toxic effects of drugs intended for use as antimalarials in man. The intended purposes of these studies are to provide a basis for predicting human response and to fulfill requirements for submission of IND for clinical trials of new antimalarials for military personnel in malarious areas.</p> <p>24. (U) The approach is to study both the effects of antimalarial drugs on healthy animals and the fate of these drugs in healthy animals in order to predict the human tolerance to new drugs (Phase I). The handling of antimalarial drugs by diseased animals is being studied to determine the effects of malaria upon pharmacokinetics. This is in order to predict the tolerance of new antimalarial drugs in human efficacy studies (Phase II).</p> <p>25. (U) 77 10 - 78 09 Technical management continued for 11 contracts in pharmacology. One new IND application and 15 supplements were written. Cardiorespiratory evaluation of WR 194,965 in dogs suggest that the negative chronotropic effect may be mediated in part by the autonomic nervous system. Validation was made of the high pressure liquid chromatography method using normal mobile phase for 3 antimalarials and blood levels of antimalarial drugs were determined for over 1000 clinical samples. Pharmacokinetic studies have been carried out in healthy human volunteers using 2 antimalarials. Tissue distribution and pharmacokinetic studies were carried out in mice using 3 radiolabeled antimalarials. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.</p>									

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1499

Project 3M162770A803 MALARIA PROPHYLAXIS

Work Unit 087 Determination of pharmacological effects of anti-malarial drugs

Investigators.

Principal: Melvin H. Heiffer, Ph.D.

Associate: Dr. R. Rozman, MAJ J. von Bredow, CPT D. Korte, Jr., Dr. H. Chung, MAJ R. Desjardins, CPT V. Jimmerson, MAJ C. Pamplin, Dr. H. Lowensohn, SP4 D. Bounds, SP4 M. Abdelrahim, SP5 J. Osuch, D. Battle, SP5 P. Egan, SP5 H. Stampfli

1. Description.

Pharmacological investigations carried out by the department continue in two broad overlapping areas. One is the effect of the body or system on the drug, i.e., absorption, distribution, biotransformation and excretion. The second is the effect of the drug on the body or system, i.e., pharmacodynamics. In addition, continuation of the development and utilization of sensitive assay methods for several of the new antimalarial drugs was emphasized.

2. Development of a multicomponent mobile phase for the HPLC analysis of candidate antimalarial agents.

a. Background:

Currently high pressure liquid chromatography (HPLC) is the most successful method of analysis of candidate antimalarial agents in biological samples.

The HPLC system is useful only if the components of the chromatogram can be separated into distinct peaks which can be quantitated. In this system of chromatography the column remains stabilized and the mobile phase is altered until proper peak separation is achieved. Alterations in the composition of most two-part normal phase systems will induce changes in the retention of both the antimalarial peak and the internal standard peak. Therefore, even though the antimalarial peak and the internal standard peak can be affected by a change in the mobile phase, it may not be possible to separate the individual peaks. The addition of another component to the mobile phase system makes possible the simultaneous separation of the chromatographic peaks.

The candidate antimalarial compound, WR 180,409·H₃PO₄ (DL-threo-α-(2-piperidyl)-2-trifluoromethyl-6-(4-trifluoromethyl-phenyl)-4-pyridinemethanol phosphate) and its internal standard, WR 184,806·H₃PO₄ (DL-2,8-bis(trifluoromethyl)-4-[1-hydroxy-3-(N-t-butylamino)propyl]-quinoline phosphate) when extracted from biological fluids, are insoluble in reverse phase HPLC solvent systems. Therefore, they must be chromatographed using normal phase solvent systems. Blood extracts of these lipid soluble, hydrophobic antimalarials possess considerable interference patterns when chromatographed using "conventional" binary normal phase solvent systems. The blood protein interference fraction can sometimes be overcome by changing solvent strengths of a binary mixture or by selecting an alternate solvent. Changing solvent strength with respect to these antimalarials induces undesirable peak selectivity.

The manipulation of a mobile phase to control separation and retention of selected peaks represents the optimum capacity of an HPLC solvent system. A mobile phase system which has been developed to control selectivity and retention of two candidate antimalarials is presented.

This versatile multicomponent mobile phase system can be utilized to analyze Walter Reed compounds WR 180,409, WR 184,806 and WR 142,490. This mobile phase system was employed to analyze WR 142,490·HCl and WR 142,490·CH₃SO₃H levels in human blood samples collected from the clinical laboratories. During the past year more than 1000 human clinical samples have been analyzed for blood levels of mefloquine.

b. Materials and methods:

The solvents hexane, ethylene dichloride, acetonitrile, and methanol were UV spectrograde (Burdick and Jackson Laboratories, Inc.). McGaw Laboratory sterile water for injection was used as a modifier, as well as reagent grade 88% formic acid (Fisher Scientific Co.). The drug, WR 180,409·H₃PO₄, and its internal standard, WR 184,806·H₃PO₄, were assayed at 98% purity. The drug solutions were made as the salts and all concentrations were expressed as the free base. Chromatographic separation was accomplished using a μ Bondapak CN column (Waters Associates, Inc.) jacketed with a temperature control block (Waters Associates, Inc.). A temperature control water bath was utilized to maintain all mobile phase systems at a constant temperature of 25.0 \pm 0.5°C. A Waters Associates Model 6000A solvent delivery system, U6K injector and Model 440 UV absorbance monitor with 280 nm filter completed the high pressure

liquid chromatography system. An Omniscribe (Houston Instruments) single channel recorder was used.

All injections were made with either a 25 μ l or a 100 μ l precision sampling syringe (Altech Associates). Filtration of solvent mixtures was conducted using two methods: qualitative filter paper (Schleicher and Schuell, Inc.); and solvent clarification kit (Waters Associates) utilizing a 0.5 μ m pore size organic solvent filtration disk.

The major and minor components of the mobile phase systems were prepared in the ratios indicated in Table 1. After the solutions were prepared, a water:formic acid solution (50:50 v/v) was added to the mixtures sufficient to saturate each mobile phase system at 25.0°C. The solutions were filtered using qualitative filter paper followed by the solvent clarification kit and placed in a temperature control water bath maintained at 25.0 \pm 0.5°C. Sufficient column volumes of mobile phase were utilized to achieve retention reproducibility on repeated injections. Standard solutions of WR 180,409 \cdot H₃PO₄ and WR 184,806 \cdot H₃PO₄ were prepared in methanol. The flow rate of the solvent delivery system was maintained at 2 ml/min and the chart speed was set at 1.25 cm/min. The volume of injection for the standard solutions was 5 μ l.

In order to achieve statistical evaluation, 4 repetitions of all procedures were conducted. The calculations for capacity (K') or retention as well as selectivity (α) were determined using standard equations. The peak area was obtained from the product of the maximum peak height (cm) and the width at peak half-height (cm). Peak areas were plotted against actual concentrations in the standard curve determinations.

The blood extract was prepared by spiking 5 ml blood samples with 50 μ l of an equimolar concentration of the anti-malarial drug and its internal standard. The blood sample was then mixed. Five ml of pH 7.4 phosphate buffer were added followed by 10 ml of ethyl acetate. The sample was then mixed for 30 min whereupon it was centrifuged for 10 min. The ethyl acetate layer was removed and evaporated to dryness using a water bath evaporator. Additional ethyl acetate was added to the blood layer and the procedure was repeated two additional times. The sample was reconstituted in 500 μ l of the corresponding mobile phase; 50 μ l of the sample was injected and chromatographed.

c. Results:

The manipulation of selectivity of the column and retention of candidate antimalarials using isocratic conditions is readily observed in Figure 1. Alterations in the ratio of the major components, ethylene dichloride and hexane, induced significant changes in retention (K') of the antimalarial and its internal standard as shown in Parts A and B of Figure 1. The addition of various concentrations of the minor component, acetonitrile, significantly altered the selectivity of the column as illustrated in Parts C, D, E and F of Figure 1.

The acetonitrile-induced alterations in selectivity of the column with respect to varying ratios of the major components, ethylene dichloride and hexane, are presented in Table 2. With alterations in the ethylene dichloride/hexane ratio the selectivity appears to have a non-linear relationship with respect to various acetonitrile concentrations.

Alterations in the ratio of major components led to shifts in polarity related to the equation $P = .01 (\%a P_a + \%b P_b + \%c P_c)$, where $\%a$, $\%b$ and $\%c$ are the percent of ethylene dichloride, hexane and acetonitrile respectively in the mixture, while P_a , P_b and P_c are polarity (solvent strength) of ethylene dichloride, hexane and acetonitrile respectively. A linear relationship can be derived from this equation as illustrated in Figure 2.

The ability to manipulate selectivity and retention is useful for the analysis of blood extracts. Protein fractions extracted from blood along with the candidate antimalarials cause considerable interference which may be overcome by altering solvent strength. When the antimalarial peaks were separated from the interference, alteration in the minor component, acetonitrile, caused an increase in selectivity, thereby optimizing the separation as is demonstrated in Figure 3.

Alterations in the components to optimize separation had no effect on linearity of the standard curve, as shown in Figure 4. In 4 significantly different mobile phase systems, the standard curves did not deviate from linearity. Means and standard deviations are given in Table 3.

d. Discussion:

Alterations in the ratio of major components of the solvent system, ethylene dichloride and hexane, induce a shift in the equilibrium equation; $X_m + n S_{ads} \rightleftharpoons X_{ads} + n S_m$. In this equation, X_m and X_{ads} represent candidate antimalarial molecules in the mobile phase and the adsorbed state respectively. Free mobile phase molecules of ethylene dichloride and hexane are represented by S_m while the mobile phase molecules adsorbed on the surface are represented by S_{ads} . The number of adsorbed solvent molecules, n , must be displaced by the adsorption of the candidate antimalarial, X . When the solvent, ethylene dichloride and hexane, interacts strongly with the surface sites, the equilibrium is shifted to the left and the candidate antimalarials remain in solution and capacity (K') decreases. Under ideal conditions, this equation is functional for alterations in the major components of the mobile phase (Figure 1: Parts A and B). The development of a tertiary mobile phase, with the addition of acetonitrile, may invalidate the above equilibrium equation.

An alteration in column selectivity was observed with small additions of the minor component, acetonitrile. Although the addition of acetonitrile induced insignificant alterations in polarity (Figure 2) which may be responsible for minor shifts in the retention of peak I (Figure 1: Parts C, D, E and F), these minor alterations in polarity may not be responsible for the significant alterations in peak II (Figure 1: Parts C, D, E and F).

The column selectivity changes observed in Table 2 demonstrate the significance of small additions of the minor component, acetonitrile, in the ability to achieve ideal separations of these candidate antimalarial compounds.

The selectivity alterations observed in Table 2 could have been caused by other variables. A great influence of small increases of water and temperature on retention has been demonstrated. The water and formic acid content were stabilized by saturation at constant temperature and therefore assumed to remain constant. The formic acid was used in addition to water to deactivate the column and to prevent peak tailing. With these variables controlled, the acetonitrile appears to be the only component which could be causing the alterations demonstrated in Figure 1 and Table 2.

The flexibility of this mobile phase system is demonstrated in Figure 3. Since protein fractions from blood extracts create considerable chromatographic interference, the manipulation of major components (ethylene dichloride and hexane) isolated the antimalarial and the internal standard fraction while simultaneous concentration changes of the minor component, acetonitrile, effected separation of the antimalarial from the internal standard. The ability to selectively control retention of the antimalarial and its internal standard facilitates the evaluation and quantitation of the resulting chromatograms.

Although significant changes in column selectivity are induced by the alteration of mobile phase systems, the standard calibration curves (Figure 4) remained linear over a wide range of mobile phase systems. The maintenance of linearity of the corresponding calibration curve enables the use of simple linear regression plots in the quantitative analysis of the selected candidate antimalarial.

3. Validation of the quantitation of mefloquine in human whole blood samples.

a. Background:

Mefloquine (WR 142,490) is currently the most promising drug for the treatment of resistant strains of malaria. Various drug formulations of mefloquine have been developed and bioavailability investigations are currently underway. The validity of these bioavailability experiments are dependent to a significant extent upon the accuracy and precision of the analytical method for mefloquine.

A high pressure liquid chromatography method of analysis was developed in this laboratory and was modified to incorporate a multicomponent mobile phase. A validation of this technique was conducted during the past year and is described in this annual report.

b. Materials and methods:

The candidate antimalarials, mefloquine·HCl (WR 142,490·HCl, Lot AS) and the internal standard, WR 184,806·H₃PO₄, Lot AJ, were assayed at 98.8% and 98.2% purity, respectively. All solvents were of spectral quality and utilized as purchased from Burdick and Jackson without further purification. The salt

form of each compound was dissolved in methanol for the production of stock solutions. All concentrations are expressed as the free base form.

A Waters Associates High Pressure Liquid Chromatograph (HPLC) equipped with a 280 nm ultraviolet absorbance monitor was utilized to assay drug standards and ethyl acetate extracts of blood samples. Standards and blood extracts were chromatographed in a Waters Associates microbondapak CN column. A mobile phase composed of two major components, hexane and ethylene dichloride, one minor component, acetonitrile, and two trace modifiers, water and 88% formic acid was adjusted to generate optimal chromatographic separations as described in Section 2 of this Annual Report.

Standard solutions of the candidate antimalarial, WR 142,490·HCl, Lot AS, and its internal standard, WR 184,806·H₃PO₄, Lot AJ, were made in methanol and chromatographed directly. Standard biological WR 142,490 solutions were produced in outdated whole "blood bank" blood. Small quantities of methanol solutions of WR 142,490 were added directly to the blood samples by means of a Precision Instruments microsyringe. The blood sample was shaken for 30 min on a shaker bath to insure uniform distribution.

The blood samples were either processed immediately or stored under frozen conditions. The general assay procedure is outlined in the flow diagram in Table 4.

c. Results and discussion:

The determination of the precision of an analytical system can best be achieved through the use of a combined or "pooled" sample. In this procedure the compound to be analyzed is added to the entire biological sample and is mixed thoroughly for uniform distribution. Ultimately each aliquot of the entire biological sample should contain the same amount of compound per unit of sample. The statistical notations and abbreviations used are indicated as follows:

$\bar{X} \pm S_m$ = statistical mean \pm standard deviation of the mean

S = standard deviation

CV = coefficient of variation = $\frac{S}{\bar{X}}$ (100)

$$\% \text{ Error} = \frac{\text{calculated concentration} - \text{actual prepared concentration}}{\text{actual prepared concentration}} (100)$$

Results of analyses of replicate "unknowns" produced from a pooled sample of WR 142,490 (concentration of WR 142,490 in whole blood unknown to the technician) are shown in Table 5.

These results suggest that there is no significant difference in precision and accuracy between whole blood samples which were maintained at room temperature and those which were frozen and then thawed before analysis.

Two sets of spiked blood samples were analyzed in a manner which would simulate the human clinical sample as closely as possible. 5.0 ml aliquots of outdated human bank blood were pipetted into "Corex" round bottomed centrifuge tubes. The blood aliquots were individually spiked with the stock methanol solutions of mefloquine. The volume of the stock solution which was added to each aliquot of blood never exceeded 0.30 ml; however, in the calculation of the actual concentration a volume correction was always included. Results of analyses of individually prepared WR 142,490 spiked blood samples are given in Table 6.

The results of pooled blood samples as well as individually spiked blood samples suggest that the present high pressure liquid chromatography procedure is accurate to + 8% at mefloquine concentrations greater than 0.150 $\mu\text{g/ml}$ and accurate to approximately + 20% at levels of 0.050 $\mu\text{g/ml}$. The precision or reproducibility of the analytical technique approximates a coefficient of variation of 10% or less over the observed range of 0.050 $\mu\text{g/ml}$ to 2.00 $\mu\text{g/ml}$.

Both the accuracy and precision of this analytical system depend on the development of a linear standard curve with which the chromatograms are quantitated. The standard curve is readily prepared by the direct injection of standardized solutions of WR 142,490 and its internal standard into the HPLC system. The standard curve may also be produced from blood extracts in order to compensate for background interference patterns; however, since the interference pattern of outdated bank blood will probably not be the same as that of the patient, a direct injection of standard solutions utilizes the best approximation available. Direct injection of methanol solutions of the standards also enables the

chromatographic system to function at near maximal efficiency; therefore, an analysis of the standard curve chromatograms should aid in determining the limits of chromatographic interpretation. In all of the chromatograms used in the development of the standard curve the internal standard peak area should remain constant; however, the coefficient of variation (CV) indicates a range of 3 to 8%. The ratio of the area of the compound to be analyzed (WR 142,490) to the area of the internal standard (WR 184,806) is more consistent (CV = 0.5 to 4%) for all but the lowest concentration (0.050 $\mu\text{g}/5 \mu\text{l}$) of WR 142,490 (CV = 19%). Chromatographic recovery of the internal standard from blood extracts indicates that the same degree of variation (CV = 2 to 8%) in the determination of peak area exists. The ratio of the area of WR 142,490 extracted from blood to the area of the internal standard extracted from blood also varied to the same extent as the ratio of the area of the standard methanol solutions (CV = 1 to 4%) in all but the lowest concentration of WR 142,490 (CV = 9%). These results suggest that the blood extracts of WR 142,490 and its internal standard, WR 184,806, are being chromatographed to a near optimal extent in this experiment.

Since all manual methods of chromatogram quantitation are approximations, improved quantitation could be achieved by means of an integrator. Although significant improvements have been made in recent years, quantitation by means of automatic integrators on anything other than perfect chromatographic peaks may be somewhat doubtful. The entire concept of the analysis of chromatograms is based on the evaluation of single, well defined peaks over a linear background. The background or "blank" can be determined by treating a blood sample containing no drug or internal standard in a manner identical to the normal procedure. Interference patterns in the chromatograms are readily visible and may sometimes be accounted for by calculating the area corresponding to the interfering peaks and simply subtracting it from the area of the drug peak. This concept may be important in the analysis of low blood concentrations of the "unknown" in which an interfering peak may readily obscure the drug peak by 30 to 50%. In acute animal experiments a "blank correction" can be employed routinely; however, in human clinical samples a "blank" sample is seldom available. During the analysis of blood samples from a long term experiment, the blank may be expected to change. Therefore, corrections made with respect to the actual time of drug administration will not be consistent throughout the analytical system.

Since there does not appear to be a significant difference between the chromatography of the standards prepared in methanol and the chromatography of the blood extracts of the same compounds, it should be possible to estimate the accuracy of the chromatographic system by the precision of the standard curve used to evaluate the blood extracts. In an ideal analytical system the standard curve should be linear and have an intercept near zero.

The development of a control system which spans the normal clinical range of drug concentrations is possible. Although the control system attempts to mimic the normal clinical blood sample and should be a more reliable comparison than the methanol standards, it cannot be the same as the clinical sample. Therefore, the possibility of accurate control values coupled with erroneous clinical sample results remains.

Since all of these studies have been developed on the basis of spiked blood samples, they are still only an approximation of the actual human blood sample. A more realistic approximation may be achieved through the collection of a large pool of clinical blood samples. This pool of human clinical blood samples must be well mixed for complete uniformity so that each aliquot will contain the same amount of drug per unit volume or per unit weight. Although the absolute concentration of the drug in each aliquot of pooled blood can not be determined, the reproducibility of the analytical system can be monitored.

This is by no means an exhaustive study of all possible methods of analysis of mefloquine (WR 142,490) in biological fluids. Experimentation will continue primarily in three areas in an attempt to improve the analytical system: 1) attempts to improve biological sample preparation "clean up" procedures will continue, 2) improved, simplified, mobile phase systems will be explored and 3) improved analysis of chromatograms will continue to be investigated.

4. Human pharmacokinetic studies.

a. Background:

During the past year a major portion of the clinical program of this department has involved the application of pharmacokinetic principles to the study of new antimalarial drugs. Development of the techniques for measuring various

drugs of interest to the program in biological fluids made possible the pharmacokinetic and bioavailability studies which have been conducted. The clinical protocols were carried out by a private contractor and will therefore not be discussed here in any detail. However, each protocol was designed and generated by the staff of this department, and since the design is critical to the final analysis of data each will be described briefly. The analysis of blood specimens for drug content was also performed by this department and the methodology is discussed elsewhere in the Annual Report. Final analysis and reporting of blood concentration-time data were performed by the clinical staff of this department.

There are several possible approaches to the generation and analysis of pharmacokinetic data. The data generated in clinical studies performed in the past were limited to whole blood concentrations of parent compounds at selected sampling times in relation to single or multiple doses of each drug. Two drugs have been investigated in 5 separate protocols. They are both quinolinemethanols: WR 142,490 (mefloquine) and WR 184,806.

One approach which was evaluated and discarded as insufficient for modern pharmacokinetic studies was the use of analogue curve fitting. Instead, the use of digital computer technology with application of iterative non-linear programming was chosen to provide the kind of statistical data required to support the purposes of the pharmacokinetic studies. A review of available pharmacokinetic programs concluded that none provided the level of sophistication and flexibility desired.

b. Methods, results and discussion:

A minicomputer with unique graphic capabilities was selected and a number of programs developed which are tailored to our particular needs. The program we are currently using was adapted to a Tektronix 4051 Graphics Computer from a Basic Program written by Homer and Horowitz of NAMRI. It employs a modified Marquardt algorithm for non-linear parameter estimation. Considerable effort was invested in designing our program to perform a number of useful functions. It first utilizes an interactive graphics system to select a variety of likely regression models to fit the data to be analyzed. Initial parameter estimates are then derived graphically and optimized by an iterative algorithm by minimization of the residual sum of squares. This parameter estimation routine has been evaluated by comparison of results obtained with the non-linear

regression program in the BMD-P run on a CDC 1500 computer. Our program also provides an option for selecting pure non-linear regression models with hybrid rate constants or equations with microrate constants based on a variety of classical compartmental models. All of the models used to date employ only first order processes. Various statistical tests and graphic displays are then used to select the model which provides an optimum fit to the particular set of data. This program and the use of a minicomputer system which is committed to pharmacokinetic data analysis has provided the department with a highly flexible and important resource in the overall development of new anti-malarial drugs.

The purposes of pharmacokinetic studies are to establish bioavailability, to optimize administration, and to gain insight concerning the time-curve of action of a particular drug. The first drug in the antimalarial drug development program to undergo detailed pharmacokinetic study was the quinolinemethanol, WR 184,806. Single oral doses of 3 to 16 mg/kg of the drug were administered to 20 volunteers. Blood samples were obtained at selected times up to 336 hr after drug administration and each was analyzed for whole blood concentration of WR 184,806 as parent compound. Measurable blood levels were achieved at all doses. In most cases the blood concentration-time data were best fit to a biexponential function describing a single-compartment open model with a "lag-time" of 1-2 hr. The mean absorption $t_{1/2}$ was 0.9 hr with a mean t_{max} of 4.3 hr. The average half-life of the drug was 25.3 hr, indicating the likelihood that a multiple dosing regimen will be required for treatment of malaria. The area under the concentration-time curve was found to be a linear function of the dose administered with intercept = 0, a slope of - 1.2277 and an $r^2 = .7325$. This suggested, but did not prove, that the drug, WR 184,806, behaved according to linear pharmacokinetics over the range of doses tested. That is to say, all absorption, distribution, metabolism and elimination processes were first order. Further studies with multiple dosing are planned.

A second pharmacokinetic study was conducted with another quinolinemethanol, WR 142,490 (mefloquine). It was known prior to this study that the drug is effective as a single oral dose in the treatment of chloroquine-resistant falciparum malaria. It was, therefore, anticipated that WR 142,490 would be well absorbed and have a rather long half-life. Single oral doses of 3 to 22 mg/kg were administered to 20 volunteers. Blood samples were obtained at selected times up to 1992 hr after drug administration and each was analyzed for whole blood

concentration of WR 142,490 as parent compound. The extended period of sampling and specific sampling times were selected from data obtained from a single volunteer, in whom the drug was shown to have a half-life of 16.5 days. In most of the 20 subjects the blood concentration-time data were best fit to a triexponential function describing a two-compartment open model with a "lag time" of about 30 min. The absorption of the drug was much faster when administered as an oral suspension ($k_a = 15.3422 \text{ days}^{-1}$) than as the tablet formulation ($k_a = 4.3711 \text{ days}^{-1}$). The mean half-life was expectedly long, 13.89 days, but had an extremely large range (6.48 to 22.65 days). The area under the concentration-time curve was found to be linearly related to the dose administered with intercept = .07, slope = 1.0 and an r^2 of .9783. This suggests that WR 142,490 also behaved according to linear pharmacokinetic principles.

A comparative bioavailability study with 2 tablet formulations of WR 142,490 was also conducted. The results suggest that one of the formulations provided more rapid absorption and higher peak blood concentrations of the drug, but the resulting areas under the concentration-time curves were not significantly different. The ratio of the fraction absorbed of one formulation to the other was ~ 1.4 . However, this was a cross-over study, and the range of ratios varied from 0.7 to 1.6. A second comparative bioavailability study comparing a Walter Reed formulation and one provided by F. Hoffman LaRoche was recently performed. This was also a cross-over study, and the data are still being analyzed.

A multiple dose safety and tolerance study involving the weekly administration of 250 mg of WR 142,490 to each of 25 volunteers was conducted. This was not designed as a pharmacokinetic study; however, several blood specimens were obtained for measurement of whole blood concentrations of the drug from each subject. The data are available and appear to fit the expected range of concentrations, but a detailed analysis of these data has not yet been made. Additional multiple dose pharmacokinetic studies with WR 142,490 are planned.

5. The absorption, distribution and excretion of WR 142,490-HCl in mice.

a. Background:

The compound d,l-erythro-2,8-bis(trifluoromethyl)- α -(2-piperidyl)-4-quinolinemethanol hydrochloride, named

mefloquine hydrochloride, has been shown to be very efficacious against human malaria, including that caused by multidrug-resistant strains. The purpose of this study was to examine the absorption, distribution and excretion of mefloquine in the mouse.

b. Materials and methods:

Mefloquine hydrochloride was synthesized by the Aerojet Solid Propulsion Co. (Sacramento, CA) and the C14-labelled mefloquine hydrochloride was synthesized by Monsanto Research Corporation, Dayton Laboratory (Dayton, OH) with the C-14 label in the methanolic carbon (specific activity either 11.5 or 12.5 mCi/mmol, radiochemical purity ~99%). The radiolabelled compound was diluted by dissolving it in methanol with the appropriate amount of nonradiolabelled drug and purified by crystallization from ethyl acetate/heptane to a final specific activity of either ~0.4 or ~4 μ Ci/mg, with a radiochemical purity of >99%. All of the above chemicals were assayed for chemical and radiochemical purity by thin-layer chromatography (TLC). All other chemicals and solvents used were of reagent grade.

Female albino ICR mice from the Walter Reed colony, weighing 25-30 g, were used for all experiments. The mice were fed a standard laboratory diet which had been molded and baked into food sticks with chicken eggs as binder. The mice were fasted for 16 hr before dosing but were allowed water ad lib. Food and water were provided ad lib immediately after dosing. The mice were housed 4 or 6 to a cage in glass modified Delmar-Roth metabolism cages without expired air traps, except where noted. All animals were dosed either perorally (po) by gavage at 8-10 mg/kg or intraperitoneally (ip) at 10 mg/kg with a suspension of radiolabelled mefloquine hydrochloride in an aqueous solution of 0.2% methyl cellulose:0.4% Tween 80®. Standard doses taken before, during and after dosing the animals were assayed for C14.

Two groups of 6 mice each were dosed either po by gavage or ip, and the urine and feces were collected separately at 12 hr intervals for 6 days and at 24 hr periods thereafter until >90% of the total radioactivity had been excreted. One group of 4 mice was also monitored for 240 hr for the presence of ¹⁴C-labelled volatiles in the expired air.

After determination of the total radioactivity content, urine samples, including appropriate cage washings, were cycled

3 times through Amberlite XAD-2 resin (5 g/50 ml) in glass columns (1.2 x 10.0 cm). The resin was washed with 3 bed volumes of water and eluted with 4 bed volumes of absolute methanol. The methanol eluates were evaporated to dryness in a flash evaporator, reconstituted in 2-5 ml of methanol, and stored at -10°C for later TLC analysis.

Fecal samples, including appropriate cage washings, were homogenized in methanol (approximately 50 ml) in a Waring Blender, slurry-packed into glass columns (1.2 x 25.5 cm) and eluted with absolute methanol (approximately 250-500 ml). After determination of total radioactivity, the methanol eluates were evaporated as above, reconstituted in 10-20 ml of methanol, and stored at -10°C for later TLC analysis.

The expired air from one cage of 4 mice was monitored for 240 hr by drawing air through the cage and then through a series of 4 traps in the following sequence: traps 1 and 2, ethanolamine/1,2-dimethoxyethane (1:2 v/v); trap 3, 2 N sulfuric acid; trap 4, 2 N sodium hydroxide. The traps were drained and refilled every 12 hr and aliquots removed for determination of total radioactivity.

Six groups of 6 mice each were dosed po by gavage at 8.4 mg/kg. Each group was anesthetized with ether at the appropriate sampling time and exsanguinated via a surgically exposed femoral artery. The groups were killed at 4, 8, 24, 48, 72 and 96 hr. Blood samples were collected in heparinized syringes. Two 100 μ l aliquots were removed from the pooled whole blood samples for determination of total radioactivity, a microhematocrit was determined (13,000 g for 3 min) and plasma separated from packed red blood cells (RBC) after centrifugation for 20 min at 2000 g. Two 100 μ l aliquots of plasma were removed for determination of total radioactivity and the remainder of the plasma and RBC samples was stored at -10°C for later extraction.

The frozen plasma and packed RBC samples were thawed and 1 to 2 ml aliquots of each placed in separate glass centrifuge tubes. Two vols of pH 7.4 Sorensen phosphate buffer (M/15) were added and 20 vols of ethyl acetate added. Extraction was by vigorous mixing for 5 min followed by centrifugation to separate the phases. The ethyl acetate extract was washed with pH 11 phosphate buffer. After centrifugation, the ethyl acetate layer was pipetted off, evaporated and the residues dissolved in methanol (0.5 ml) and stored at -10°C for later TLC analysis.

Two groups of 4 mice each were dosed po by gavage at 10 mg/kg and one group was killed at 24 hr and the other at 48 hr. At the time of death the mice were anesthetized with ether, exsanguinated via a surgically exposed femoral artery and various organs were removed. The organs were pooled by tissue type at each kill time and stored in methanol at -10°C, as were the residual carcasses. Urine and fecal samples were collected separately until the time of death.

The tissue pools were homogenized and extracted as described above. The methanol extracts were evaporated as described above, reconstituted in methanol (0.5-2.0 ml) and stored at -10°C for later TLC analysis. Carcasses were homogenized in methanol with a tissue grinder and treated as described for the other tissue samples.

Appropriately sized aliquots were removed from the samples during the various procedures to monitor the recovery of total radioactivity. The recovery of radioactive components derived from mefloquine-C14 from the various sample types by the methods described was shown to be 95-100% efficient in all cases.

A Packard TriCarb liquid scintillation counter, model 3320, or a Searle Mark II liquid scintillation counter, was used to count all samples. The scintillation solution was either a mixture of 2,5-diphenyloxazole in toluene (6 g/liter) and 1,2-dimethoxyethane (5:3, v/v) or a premixed scintillation fluid (Hydromix®). Whole blood, plasma, and packed red blood cell samples were digested in 0.2 ml of 70% perchloric acid and 0.4 ml of 30% hydrogen peroxide with heating at 75-80°C for 1 hr. Counting efficiency was determined by means of either an internal or an external standard.

All chromatograms were developed for 10 cm beyond the origin on silica gel F254 precoated glass plates with one of the following solvent systems: A) heptane/1-butanol/glacial acetic acid (8:1:1, v/v/v) with six serial developments; and B) 1-butanol/glacial acetic acid/water (66:17:17, v/v/v). After development, the plates were air-dried, visualized with UV light (254 nm), and the radioactivity localized and quantitated using a Varian Model 6000 two-dimensional radiochromatogram scanner with integrator. Samples were applied as 1-cm to 4-cm bands with a 2-cm reference band of mefloquine on every plate.

For inverse isotope dilution, appropriate aliquots of 24-36 hr urines and 24-36 hr fecal extracts were dissolved

separately in methanol (10 ml) and nonradioactive mefloquine hydrochloride (~100 mg) added. After thorough mixing, the solutions were evaporated to dryness in a flash evaporator. The residues were extracted with hot ethyl acetate and the extracts evaporated. The resultant precipitates were repeatedly recrystallized from ethyl acetate:heptane until a constant specific activity and melting point had been achieved twice in succession.

To determine presence of conjugates, hydrolysis of urine and fecal extracts was attempted. Aliquots of pooled 0-96 hr urine and fecal extracts were incubated at 25°C for 20 hr in one of the following media: a) control, deionized water; b) bovine β -glucuronidase, 1760 Fishman Units in deionized water; c) β -glucuronidase/arylsulfatase (*Helix pomatia*), 55,000 Fishman Units and 29,000 Fishman Units respectively in deionized water; d) phenolphthalein glucuronide or phenolphthalein sulfate added to the appropriate biological extracts in deionized water as positive controls. After incubation, the positive controls were made alkaline with sodium hydroxide to prove enzyme activity under the experimental conditions. The other samples were evaporated to dryness in a flash evaporator with the aid of added ethanol and the residues extracted with methanol. Aliquots were removed for determination of total radioactivity and the remainder subjected to TLC analysis.

c. Results:

The primary route of excretion of radioactivity derived from mefloquine hydrochloride was via the feces after either po or ip administration of 10 mg/kg (Table 7). Urinary excretion of C14-drug equivalents was appreciable (~20%) but only trace amounts of radioactive volatiles were detected in the expired air.

TLC profiles using solvent A contained 5 major and at least 3 minor radioactive peaks. The parent drug usually exhibited an R_f value of 0.24. The TLC profiles using solvent B contained 3 major and at least 3 minor radioactive peaks. The parent drug usually exhibited an R_f value of 0.73. The percent of unchanged drug as calculated from the TLC scan correlated closely with the percent of unchanged drug as determined by inverse isotope dilution.

As shown in Table 7, the percentage of parent drug decreased with time in both urine and feces. This was the case

whether drug was administered po or ip. Approximately 30% of the dose was excreted as parent drug after either route of administration. The elimination half-times for the parent drug were calculated using the sigma-minus method. The half-times of elimination via the excreta were: 18.7 hr for urine and 14.5 hr for feces after po dosing; 13.5 hr for urine and 15.4 hr for feces after ip dosing.

Representative aliquots of urine and fecal extracts were subjected to enzymatic hydrolysis with either β -glucuronidase or β -glucuronidase/arylsulfatase. These treatments did not cause any significant changes in the chromatographic profile or the relative composition of radioactivity as analyzed by TLC. Positive controls incubated concurrently showed that the experimental conditions used were capable of producing full enzymatic hydrolysis.

The plasma concentration curves for mefloquine-C14 and total C14-drug equivalents, expressed as μ g of drug equivalents per ml, are summarized in Table 8. The highest level of total radioactivity in the plasma was observed at 24 hr while that of parent drug was observed in the first sample (4 hr). In the RBC, the peak concentrations of total radioactivity and of parent drug were seen at the 4 and 8 hr time points. The concentrations of mefloquine in the RBC were about 5-6 times those in the plasma. When concentrations were plotted vs. time semi-logarithmically, linear regression analyzed and the slope of the line determined, the elimination half-lives of the parent drug in plasma and in RBC were calculated to be 17.0 and 18.6 hr respectively.

The distribution of mefloquine-C14 in selected tissues was studied (Table 9). Of the tissues investigated, the major sites of distribution of mefloquine-derived radioactivity were the liver, lungs, kidneys and residual carcass, with large amounts in the gastrointestinal tract plus their contents, especially the small intestine. A high percentage of parent drug was present in the tissue extracts as identified by TLC. However, the heart contained primarily metabolites at both 24 and 48 hr. The bile contained a high ratio of metabolites to unchanged compound, but this was not reflected in the ratios found for small intestine plus contents. The ratios found in the cecum and large intestine, plus their contents, favored metabolites over parent compound.

d. Discussion:

Mefloquine is a quinolinemethanol derivative with very good antimalarial properties. The drug was very well absorbed po by mice at doses of 10 mg/kg, as shown by the almost identical excretory pattern to that seen after ip administration. The primary route of excretion was fecal, indicating biliary and possibly gastric secretion. This interpretation is in keeping with the amounts of radiolabel found in the bile, small intestine (including contents) and the stomach (including contents) at 24 and 48 hr after dosing. In addition, other workers found that bile-fistula rats excreted a mean of 56% of the radiolabel in bile within 48 hr of a 7 mg/kg ip dose, with 9% still being excreted in the feces during that time period. They also showed that drug-derived radioactivity was secreted into the gastric juice of rats. With a pKa of 8.6, gastric secretion of this base is not surprising; quinine (a quinoline-methanol with a pKa of 8.4) has been shown to be excreted into gastric juice.

The concentration ratio of mefloquine in mouse RBC/plasma was approximately 5-6. In vitro studies by other workers produced ratios of 3.2, 3.1 and 1.7 in rat, dog and human respectively. The percent of drug-derived radioactivity present as parent drug fell much more rapidly in mouse plasma than in rat plasma. Another difference between the two species was the smaller percent of dose excreted in the urine of the rat (3.4%) than of the mouse (~20%). Another important species difference is that the rat appeared to excrete virtually no parent drug while the mouse excreted ~30% of the dose as unchanged drug.

Concentration of drug-equivalents in selected tissues was qualitatively similar in the two rodent species. Both the mouse and the rat had high concentrations of drug equivalents in the lungs, liver, kidneys and gastrointestinal tract.

6. The disposition of DL-threo- α -(2-piperidyl)-2,8-bis(tri-fluoromethyl)-4-quinolinemethanol hydrochloride (WR 177,602·HCl) in mice.

a. Background:

The quinolinemethanol derivative, WR 177,602, is a diastereoisomer of mefloquine. WR 177,602 is 27 times more potent than quinine against Plasmodium berghei malaria infections when administered orally to the mouse. This compound

is also effective in curing human and animal multidrug-resistant strains of malaria. The objective of this study was to determine the absorption, distribution, pharmacokinetics and excretion of this compound in mice.

b. Materials and methods:

Radioactive WR 177,602-HCl (Sp. Act. 32.3427 $\mu\text{Ci}/\text{mg}$; Lot No. 469a-3-1) was synthesized by Monsanto Research Corp. (Dayton, OH) with the ^{14}C -label in the alcohol carbon (Figure 5). The nonradioactive compound (Lot AD, Bottle No. BE77728) was prepared by Cordova Chemical Co. (Sacramento, CA). Radioactive and nonradioactive compounds were cocrystallized to obtain a specific activity of 1.62 $\mu\text{Ci}/\text{mg}$. Radiochemical purity of the compound was assayed by thin-layer chromatography. A suspension of the resulting mixture of WR 177,602- ^{14}C -HCl was prepared in 0.2% methylcellulose and 0.4% Tween[®] 80 in deionized water, 1 mg/ml. A premixed liquid scintillation solution, Hydromix[®] (Yorktown Research Co., S. Hackensack, NJ) was used for radioassay. All other chemicals and solvents used were reagent grade quality.

Albino, ICR female mice from the Walter Reed Colony [Walter Reed Medical (Institute of Cancer Research) Barrier-Reared mice], weighing about 25 g, were used. The mice were fed D and G Laboratory diet (G.L. Baking Co., Frederick, MD), and were maintained in a temperature controlled room with a 12-hr light-dark cycle. The mice were fasted for about 18 hr prior to dosing but were permitted water ad libitum. A dose of 10 mg/kg was administered to each mouse by oral intubation. The mice were then housed 4 to a modified Roth metabolism cage and were allowed water ad libitum. Food was not allowed until 4 hr after dosing. Standard doses were taken for radioassay before, during and after dosing the animals.

Urine samples were collected every 12 hr for 3 days and every 24 hr thereafter for 3 more days. Aliquots of each sample were taken for total radioactivity determination. The samples were lyophilized to dryness and then extracted with absolute methanol. Volumes of the extracts were determined and aliquots were taken for radioassay. The extracts were evaporated to dryness in a flash evaporator at $\sim 32^\circ\text{C}$ and reconstituted with small amounts of absolute methanol; aliquots were taken for radioassay and the samples stored in a freezer at -100°C for TLC and hydrolysis studies.

Fecal samples were collected at the same time intervals as the urine samples. The cages were washed with methanol and the washing was added to the fecal sample which was then homogenized in absolute methanol in a Waring blender and extracted in glass columns with the same solvent. Volumes of the extracts were measured and aliquots were taken for total radioactivity determination. The methanol extracts were evaporated to dryness as above and reconstituted in small amounts of absolute methanol. Aliquots were taken for radioassay and the samples stored as described above.

Plasma and red blood cell samples were collected. At various times after dosing, the 4 mice in a group were anesthetized with ether and exsanguinated via a surgically exposed femoral artery. Aliquots of the heparinized pooled blood were used for radioassay along with aliquots for hematocrit (Hct) determination (13,000 g for 3 min). The remainder of each sample was centrifuged at 7000 g for 5 min to separate the plasma from the red blood cells (RBC). Aliquots of plasma were taken from each sample for radioassay. Red blood cell drug-equivalent levels were calculated from the following equation:

$$[RBC] = \frac{[WB] - [P] (1 - Hct)}{Hct}$$

where [RBC], [WB], and [P] represent the concentrations ($\mu\text{g/ml}$) in RBC, whole blood, and plasma, respectively. The plasma and red blood cells were lyophilized and extracted with methanol as described above.

Tissue samples were isolated. At various times after dosing, all 4 mice in a group were anesthetized with ether and exsanguinated, and various tissues removed by dissection. Each tissue type from each time-interval group was pooled, placed in a preweighed container, weighed, and sufficient methanol was added to cover the tissues. The carcasses were frozen for later processing.

Each tissue pool was homogenized in methanol in a Waring blender, and the homogenate was packed into a glass column. After the homogenate was eluted and the eluate recycled, 3-4 bed-volumes of additional methanol were passed through slowly. The volume of the combined methanol eluates for each sample was measured and aliquots were taken for radioassay. The remaining eluates were evaporated to dryness and reconstituted with small amounts of methanol. Again samples were taken for radioassay and the remainders were saved in a freezer for TLC analysis.

The animal carcasses were homogenized in methanol in a tissue grinder (Model SD 45, Tekmar Co., Cincinnati, OH). The homogenates of the carcasses were processed like the tissue homogenates described above.

For radioassay, 15 ml of premixed scintillation fluid (Hydromix®) were added to each sample. The samples were counted for 10 min in a Searle Mark II liquid scintillation counter. Quenching and counting efficiency were corrected by external standardization.

Thin-layer chromatography was used to obtain metabolic profiles. Appropriate amounts of samples were streaked on EM precoated silica gel G F254 TLC plates, 0.25 mm thickness (EM Laboratories, Elmsford, NY), and developed for 10 cm from the origin with n-butanol/acetic acid/water (10:1:1) as the solvent system. This solvent was the best among 7 TLC solvent systems tested prior to the study. After air-drying, the plates were visualized with ultraviolet light (254 nm) and scanned with a Varian Model 6000 Radioscanner with integrator (time constant, 1 sec; speed 10 cm/hr; attenuation, 10 cps). The integration of the radioscanner provided a means for measuring the relative percentage of radioactivity for each peak. A control streak of ¹⁴C-WR 177,602-HCl was placed on each plate as an R_f comparison standard.

Partition coefficients were determined using various solvents. A solution of ¹⁴C-labeled WR 177,602-HCl (500 µg/ml, 32.3427 µCi/mg) was prepared in M/15 phosphate buffer (pH 7.4). Aliquots of this solution were taken to determine the total radioactivity. Duplicate 2 ml aliquots of this solution were shaken with 2 ml of various organic solvents for 2 hr at 25°C. After centrifugation, aliquots of both phases were analyzed for radioactivity. K_p was calculated as the ratio of radioactivity in the organic phase to that in the buffer phase.

Plasma protein binding was investigated. Blood was collected from the mice as described previously. Aliquots of the pooled blood were taken for hematocrit determinations. The blood was centrifuged and the plasma was separated as described above. An aliquot of a solution of WR 177,602-¹⁴C was added to 0.2 M phosphate buffer (pH 7.4) such that the final concentration of drug was 1,500 ng(base)/ml. From this stock solution, solutions with concentrations of 70, 140, 280, and 420 ng(base)/ml were made. Aliquots (1 ml) of each solution were subjected to equilibrium dialysis against the plasma. The samples were

incubated for 20 hr at 25°C in a Dubnoff metabolic shaker at 100 strokes per min. Radioactivity was determined from the cells on both sides of the membrane and the percentage of drug bound to plasma proteins calculated from the following equation:

$$\% \text{ bound} = \frac{[\text{Prot}] - [\text{buff}]}{[\text{Prot}]} \times 100$$

where [Prot] and [buff] are the radioactivities (dpm/ml) on the protein and buffer sides of the membrane, respectively.

c. Results:

The disposition of WR 177,602·HCl was studied over a 160 hr period after 10 mg of the ¹⁴C-labelled drug was administered per kg orally to mice. About 70% and 23% of the administered radioactivity were excreted via feces and urine, respectively (Table 10). The excretion of the radioactivity in urine and feces peaked in the time period between 24 and 36 hr. Approximately 2.10% of the administered radioactivity was recovered in the carcasses. A total of about 95% of the administered dose was recovered from the mice.

Residual urinary and fecal radioactivities were calculated. The half-life ($t_{1/2}$) for the elimination of radioactivity in urine and feces was determined using linear regression analysis of residual radioactivity versus time plots or the sigma-minus method (Gibaldi and Perrier, 1975). Graphical results for urine and feces are shown in Figures 6 and 7, respectively. The $t_{1/2}$ for the elimination of radioactivity in urine was estimated to be 13.26 hr and the $t_{1/2}$ for the elimination of radioactivity in feces was estimated to be 13.46 hr.

The concentrations in the whole blood, plasma and red blood cells were plotted versus time. The concentration was calculated as total ¹⁴C-drug equivalents per ml. The curves are shown in Figure 8. The logarithmic values were analyzed by linear regression. The slopes of the lines were determined; the elimination half-lives of the whole blood, plasma and red blood cells were estimated to be 19.45 hr, 20.30 hr and 18.11 hr, respectively.

The amounts of parent drug in the plasma per time period were determined by TLC and radioscanner. The results were plotted (Figure 9) and analyzed in the same manner as above. The elimination half-life of the parent drug in plasma was

estimated to be 16.07 hr. There was about 82% unchanged WR 177,602 in the plasma even at 48 hr after dosing (Table 11).

The distribution of WR 177,602- ^{14}C in selected tissues was studied. Of the tissues investigated, the major sites of distribution of WR 177,602-derived radioactivity were the lungs, liver, kidneys, gastrointestinal tract plus its contents and residual carcasses 2 hr after oral administration of the drug (Table 12). Based on $\mu\text{g } ^{14}\text{C}$ -drug equivalents per gram tissue, the major sites of concentration of WR 177,602-derived radioactivity were the lungs, G.I. tract plus its contents, gall bladder plus bile, kidneys, liver, spleen, submaxillary salivary glands and eyes 2 hr after oral administration of the drug. At this time a total of 67% of the administered dose was accounted for in the body, excluding the G.I. tract with its associated contents. This indicates that at least 67% of the dose was absorbed by 2 hr after oral administration of the drug. Twenty four hr after dosing, relatively high amounts of radioactivity were distributed in the liver and substantial amounts of the radioactivity were found in the G.I. tract with contents and in the carcasses. In terms of concentration, 24 hr after dosing, the tissues with relatively high concentrations of ^{14}C -drug equivalents were the G.I. tract with contents, eyes, heart, liver and kidneys. Gall bladder plus bile and lungs had the highest concentration of all the tissues examined 24 hr after dosing. Seventy two hr after dosing, there was relatively little or no ^{14}C -drug equivalents radioactivity remaining in the tissues examined.

The lipophilic characteristic of this drug was estimated by studying its partition between M/15 phosphate buffer (pH 7.4) and various organic solvents. The results of this study, presented in Table 13, indicate that WR 177,602 has high lipid solubility.

The plasma protein binding of WR 177,602-HCl was studied by equilibrium dialysis against fresh mouse plasma. The extent of binding over the concentration range of 70 ng(base)/ml to 420 ng(base)/ml was 99%.

d. Discussion:

The fate of WR 177,602-HCl, a promising candidate anti-malarial drug, was investigated in female mice. At least 67% of the dose was absorbed by 2 hr after oral administration of the drug. It appeared that biliary excretion and/or possibly

enterohepatic circulation of this drug was taking place, inasmuch as relatively high concentrations of ^{14}C -drug equivalents in the gall bladder and bile and in the liver, as well as a high percent of the dose in the liver, of mice at 2, 6 and 24 hr after oral administration were seen. In addition, the principal route of excretion of drug-derived radioactivity was via the feces.

It may be of interest to note that the elimination half-life of the radioactivity in the feces was essentially the same as that of the urine. In addition, the elimination half-life of the radioactivity in the whole blood was the same as those of the plasma and RBC. Furthermore, there was about 82% parent drug in the plasma even at 48 hr after dosing and the elimination half-life of the parent drug in the plasma was only 4 hr shorter than that of the total radioactivity in the plasma.

The results of this study suggest that this drug appeared to be readily absorbed after being administered orally to the mice. Once it was absorbed it was extensively bound to plasma protein and other tissues.

7. The disposition of 3-di-n-butylamino-1-[2,6-bis(4-trifluoromethylphenyl)-4-pyridyl]-propanol methanesulfonate (WR 172,435- $\text{CH}_3\text{SO}_3\text{H}$) in mice.

a. Background:

A pyridinemethanol derivative, WR 172,435 methanesulfonate, is a promising candidate antimalarial drug. This compound is 27 times more potent than quinine against *Plasmodium berghei* malaria infection when administered orally to the mouse. This compound is also effective in curing human and animal multidrug-resistant strains of malaria. The objective of this study was to determine the absorption, distribution, metabolic profile and excretion of this compound in mice.

b. Materials and methods:

Radioactive WR 172,435- $\text{CH}_3\text{SO}_3\text{H}$ (Sp Act 17.8091 $\mu\text{Ci}/\text{mg}$; Lot No. 496 -3a) was synthesized by Monsanto Research Corp. (Dayton, OH) with the ^{14}C label in the alcohol carbon (Figure 10). The nonradioactive compound (Lot AK, Bottle No. BE32210) was prepared by Ash Stevens, Inc. (Detroit, MI). Radioactive and nonradioactive compounds were cocrystallized to obtain a suitable specific activity. Radiochemical purity of the compound was assayed by thin-layer chromatography. A suspension

of the resulting mixture of WR 172,435- ^{14}C - $\text{CH}_3\text{SO}_3\text{H}$ was prepared in 0.2% methylcellulose and 0.4% Tween $^{\text{®}}$ 80 in deionized water with a resulting specific activity of 3 $\mu\text{Ci}/\text{mg}$. A premixed liquid scintillation solution, Hydromix $^{\text{®}}$ (Yorktown Research Co., S. Hackensack, NJ) was used for radioassay. All other chemicals and solvents used were reagent grade quality.

Albino, ICR male mice from the Walter Reed Colony [Walter Reed Medical (Institute of Cancer Research) Barrier-Reared mice], weighing about 25 g, were used. The mice were fed D and G Laboratory diet (G.L. Baking Co., Frederick, MD), and were maintained in a temperature controlled room with a 12-hr light-dark cycle. The mice were fasted for about 18 hr prior to dosing but were permitted water ad libitum. A dose of 20 mg/kg was administered to each mouse by oral intubation. The mice were then housed 4 to a modified Roth metabolism cage and were allowed water ad libitum. Food was not allowed until 4 hr after dosing. Standard doses were taken for radioassay before, during and after dosing the animals.

Urine samples were collected every 12 hr for 5 days and every 24 hr thereafter for 3 more days. Aliquots of each sample were taken for total radioactivity determination. The samples were lyophilized to dryness and then extracted with absolute methanol. Volumes of the extracts were determined and aliquots were taken for radioassay. The extracts were evaporated to dryness in a flash evaporator at 32°C and reconstituted with small amounts of absolute methanol, aliquots were taken for radioassay and the samples stored in a freezer at -10°C for TLC and hydrolysis studies.

Fecal samples were collected at the same time intervals as the urine samples. The cages were washed with methanol and the washing was added to the fecal sample, homogenized in absolute methanol in a Waring blender and extracted in glass columns with the same solvent. Volumes of the extracts were measured and aliquots were taken for total radioactivity determination. The methanol extracts were evaporated to dryness as above and reconstituted in small amounts of absolute methanol. Aliquots were taken for radioassay and the samples stored as described above.

The expired air of the animals was scrubbed by drawing air through the cage and then through a series of 4 traps in the following sequence: a) ethanolamine/1,2-dimethoxyethane (1:2, v/v), two traps; b) 2N H_2SO_4 (aq.); c) 2N NaOH (aq.).

The traps were drained and refilled every 12 hr for 168 hr. Volumes of each trap were measured and aliquots were taken for radioassay.

Plasma and red blood cell samples were collected. At various times after dosing, the 4 mice in a group were anesthetized with ether and exsanguinated via a surgically exposed femoral artery. Aliquots of the heparinized pooled blood were used for radioassay along with aliquots for hematocrit (Hct) determination (13,000 g for 3 min). The remainder of each sample was centrifuged at 7000 g for 5 min to separate the plasma from the red blood cells. Aliquots of plasma were taken from each sample for radioassay. Red blood cell drug levels were calculated from the following equation:

$$[RBC] = \frac{[WB] - [P] (1 - Hct)}{Hct}$$

where [RBC], [WB], and [P] represent the concentrations ($\mu\text{g/ml}$) in RBC, whole blood, and plasma, respectively. The plasma and RBC were lyophilized and extracted with methanol as described above.

Tissue samples were isolated. At various times after dosing, all 4 mice in a group were anesthetized with ether and exsanguinated, and various tissues removed by dissection. Each tissue type from each time-interval group was pooled, placed in a preweighed container, weighed, and sufficient methanol was added to cover the tissues. The carcasses were frozen for later processing.

Each tissue pool was homogenized with methanol in a Waring Blendor, and the homogenate was packed in a glass column. After the homogenate was eluted and the eluate recycled, 3-4 bed-volumes of additional methanol were passed through slowly. The volume of the combined methanol eluates for each sample was measured and aliquots were taken for radioassay. The remaining eluates were evaporated to dryness and reconstituted with small amounts of methanol. Again samples were taken for radioassay and the remainders were saved in a freezer for TLC analysis.

The animal carcasses were homogenized in methanol in a tissue grinder (Model SD 45, Tekmar Co., Cincinnati, OH). The homogenates of the carcasses were processed like the tissue homogenates described above.

Hydrolysis of excreta extracts was tried. Since the total radioactivity excreted in urine was only 0.72% of the administered dose, the urine samples were not used for the hydrolysis study.

There was sufficient radioactivity excreted in the feces so that several representative fecal samples with sufficient radioactivity were chosen, namely the 24, 36, 48, 72 and 120 hr fecal samples. The fecal samples were pooled. The supernatant from the pooled sample was dried by flash evaporation. The evaporated sample was then reconstituted with 15 ml of methanol. Duplicate 0.1 ml samples were taken for radioassay. Appropriate amounts of the reconstituted sample, along with the radioactive and nonradioactive WR 172,435-CH₃SO₃H standards were streaked onto TLC plates. The plates were developed with BuOH:HOAc:H₂O (10:1:1).

Aliquots of the reconstituted sample were placed in separate 25 ml Erlenmeyer flasks. The methanol in each flask was evaporated to dryness before the following incubation media were added to separate flasks: (1) phosphate buffer (pH 5.5) as control; (2) 1700 units of β -glucuronidase (Calbiochem, La Jolla, CA) in phosphate buffer (pH 5.5); (3) 58,000 units of β -glucuronidase/arylsulfatase (Calbiochem, La Jolla, CA) in phosphate buffer (pH 5.5); and (4) 0.2 N HCl. Three positive controls were also set up: (1) phenolphthalein glucuronide + β -glucuronidase/aryl sulfatase; (2) phenolphthalein glucuronide + 0.2 N HCl; and (3) phenolphthalein disulfate + 0.2 N HCl.

The flasks were incubated at 20°C for 20 hr. At the end of incubation, each sample containing the fecal extract was extracted 3 times with 15 ml portions of diethyl ether. The ether extracts of each sample were combined and were evaporated to dryness in a gentle stream of nitrogen. The samples were then reconstituted with 1 ml of methanol. Aliquots of 0.1 ml samples were taken for radioassay and the remainders were used for TLC analysis as described above. For the positive controls, a few drops of 0.2 N NaOH were added. The appearance of a purple color indicated a positive hydrolysis process since unconjugated phenolphthalein was released.

For radioassay, 15 ml of premixed scintillation fluid (Hydromix®) were added to each sample. The samples were counted for 10 min in a Searle Mark II liquid scintillation counter. Quenching and counting efficiency were corrected by external standardization.

Thin-layer chromatography was used to obtain metabolic profiles. Appropriate amounts of samples were streaked on EM precoated silica gel F254 TLC plates (EM Laboratories, Elmsford, NY) and developed for 10 cm from the origin with n-butanol/ acetic acid/water (10:1:1) as the solvent system. This was the best system among 7 other TLC solvent systems tested prior to the study. After air-drying, the plates were visualized with ultraviolet light (254 nm) and scanned with a Varian Model 6000 Radioscanner with integrator (time constant, 1 sec; speed 10 cm/hr; attenuation, 10 cps). The integration of the radioscanner provided a means for measuring the relative percentage of radioactivity for each peak. A control streak of ^{14}C -labeled WR 172,435- $\text{CH}_3\text{SO}_3\text{H}$ was placed on each plate as an R_f comparison standard.

c. Results:

The disposition of WR 172,435- $\text{CH}_3\text{SO}_3\text{H}$ was studied over a 192 hr period after 20 mg of the ^{14}C -labelled drug was administered per kg orally to mice. About 76.4% and 0.72% of the administered radioactivity were excreted via feces and urine, respectively (Table 14). The excretion of the radioactivity in urine and feces peaked within the first 12 hr time period after administration of the drug. Approximately 19% of the administered radioactivity was recovered in the carcasses. A total of about 96% of the administered dose was recovered from the mice. Only trace amounts (0.08%) of radioactivity were detected in the expired air.

Residual urinary and fecal radioactivities were calculated. The half-life ($t_{1/2}$) for the elimination of radioactivity in urine and feces was determined using linear regression analysis of residual radioactivity versus time plots or the sigma-minus method. Graphical results for urine and feces are shown in Figure 11 and Figure 12, respectively. The $t_{1/2}$ for the elimination of radioactivity in urine was estimated to be 113.9 hr. The elimination of ^{14}C radioactivity in the feces appeared to be biphasic. The elimination $t_{1/2}$ of the first phase was 11.2 hr and 81 hr for the terminal phase. Figure 13 shows a sigma minus plot of the elimination of parent drug in feces vs. time. The elimination of parent drug in the feces appeared to be monophasic. The elimination $t_{1/2}$ was 23.6 hr. Only one major metabolite was detected in the feces by TLC and the metabolite detected apparently did not appear to be conjugated under the hydrolysis systems tested (Table 15).

Drug levels in plasma and red blood cells were determined. The concentrations of the drug equivalent radioactivity in the plasma and red blood cells are shown in Table 16. Peak levels of the radioactivity in the plasma were observed at 1.5 hr after dosing. For the red blood cells, it was 1 hr after dosing. The results were subjected to nonlinear regression analysis. The estimated $t_{1/2}$ of the absorption phase was 0.8 hr for plasma and 1.3 hr for red blood cells. For the distribution phase, the $t_{1/2}$ for plasma was 1.7 hr and no distribution phase was seen for red blood cells. In the elimination phase the $t_{1/2}$ for plasma was 49.6 hr and for red blood cells was 25.6 hr.

The distribution of WR 172,435- ^{14}C in selected tissues was studied. Of the tissues investigated, the major site of distribution of WR 172,435-derived radioactivity was the liver throughout the time periods tested (Table 17). A high percentage of the radioactivity in the liver samples was identified by TLC as parent drug. Even 120 hr after dosing the parent drug detected in the liver was 70% of the liver sample. There were substantial amounts of radioactivity in the GI tract and in the residual carcasses. Except for gall bladder, 70 to 98% of the radioactivity in the organ and tissue samples 120 hr after dosing was identified as parent drug. Based on $\mu\text{g } ^{14}\text{-C}$ -drug equivalent per gm tissue, the major sites of concentration of WR 172,435-derived radioactivity were the GI tract plus contents, gall bladder, liver, adrenal glands, spleen, lungs, kidneys and heart 2 hr after dosing. There was some shifting in concentration during the 6, 24 and 72 hr periods. However, 120 hr after dosing, the eyes, submaxillary salivary glands, lungs, liver, adrenal glands, kidneys and abdominal fat seemed to retain relatively higher amounts of the radioactivity than the other tissues examined (Table 18).

d. Discussion:

The disposition of WR 172,435- $\text{CH}_3\text{SO}_3\text{H}$ was investigated in male mice. Two hr after dosing about 12% of the administered dose was accounted for in the body, excluding G.I. tract with its associated contents. Approximately 70% of the administered dose was accounted for in the body 6 hr after dosing. This indicates that by 6 hr after oral administration of the drug, at least 70% of the dose had been absorbed by the mice. The radioactivity was distributed throughout the animal body with a relatively large amount found in the liver. The concentration of drug equivalents remained quite high in the majority of the tissues studied and a high percentage of the radio-

activity in the tissue samples was identified as unchanged WR 172,435. The results suggest that WR 172,435 deposited in various tissues after its distribution even though the percent of total radioactivity in each tissue was relatively small. Because of the tissue storage and the slow release, the elimination $t_{1/2}$ of the radioactivity in urine and feces were rather long.

8. Characterization of the cardiovascular responses of WR 194,965-H₃PO₄ following intravenous administration.

a. Background:

4-(t-butyl)-2-(t-butylaminomethyl)-6-(4-chlorophenyl) phenol phosphate (WR 194,965-H₃PO₄) is the first compound from a new class of compounds called "phenolic phenols" that has been proposed for clinical trial in the Antimalarial Drug Development Program. Initial studies in the anesthetized dog indicated that bolus injections of WR 194,965-H₃PO₄, 30 mg/kg, produced an acute but transient hypotension and reflexive tachycardia followed by a long-lasting bradycardia. Infusion of the same dosage regimen over a one hr period eliminated the hypotension reflexive tachycardia response but did not affect the bradycardia which was maintained for 2 hr following termination of the infusion. Since quinidine (Moe and Abildskov, 1975), the dextro isomer of quinine, and many antimalarials (Arora and Lal, 1963) produce cardiovascular alterations both directly and also indirectly via an interaction with the autonomic nervous system, the present study was undertaken to ascertain whether the cardiovascular alterations produced by WR 194,965-H₃PO₄ could be modified by procedures designed to interrupt the activity of the autonomic nervous system.

b. Materials and methods:

Twenty-five beagle dogs, weighing from 8.2-14.1 kg, were anesthetized with 30 mg/kg iv sodium pentobarbital. An endotracheal tube was positioned to allow monitoring of respiration via a pneumotachometer. A catheter was placed in the ascending aorta via the left femoral artery for monitoring both pulsatile and mean arterial pressure. A catheter was also placed in the left femoral vein for administration of drugs. Lead II of the electrocardiogram was recorded and heart rates determined via an integrating cardiometer. Temperature was monitored via a rectal thermistor probe and maintained near normal with a heating pad. Arterial pressure,

mean arterial pressure, respiration, the Lead II electrocardiogram and heart rate were recorded on a Hewlett-Packard Model 7758A recorder.

The beagles were divided into 5 groups of 5 animals each. The first group was the control group which received 15 mg/kg (base) of WR 194,965-H₃PO₄ (lot AG, bottle no. BG56327) given iv over a one min period and was then observed for one hr. Data were tabulated immediately prior to administration of WR 194,965-H₃PO₄ and 1, 5, 10, 20, 30, 40, 50 and 60 min following initiation of the WR 194,965-H₃PO₄ injection. The animals in the second group were pretreated with 1 mg/kg (base) atropine sulfate 10 min prior to the WR 194,965-H₃PO₄ administration. The animals in the third group were bilaterally vagotomized 15 min prior to administration of WR 194,965-H₃PO₄. The fourth group received hexamethonium HCl 10 mg/kg (base) 15 min prior to receiving WR 194,965-H₃PO₄. The fifth group received 2 mg/kg propranolol HCl 10 min prior to the WR 194,965-H₃PO₄ injection. The hexamethonium, atropine and propranolol were dissolved in isotonic saline while WR 194,965-H₃PO₄ was dissolved in ethanol:5% aqueous dextrose (10:90, v/v).

Heart rate, mean arterial pressure and respiratory rate at 1, 5, 10, 20, 30, 40, 50 and 60 min after WR 194,965-H₃PO₄ injection were tabulated as a percentage of the baseline, the immediate pre WR 194,965-H₃PO₄ injection value. Dunnett's test of significance (Zar, 1974) was used to compare values at the various recording intervals with baseline and also to compare the effect of the different pretreatment regimens with the control WR 194,965-H₃PO₄ injection at each recording interval. The 5% level of significance was used.

c. Results:

WR 194,965-H₃PO₄, 15 mg/kg (base), injected intravenously over a one min period, produced a marked drop (58% of baseline) in blood pressure from which the animal had essentially recovered 5 min after the injection (Figure 14). Associated with the hypotension was a period of increased respiratory rate which was not statistically significant due to the large individual variation (Table 19). The WR 194,965-H₃PO₄ injection also produced a decrease in heart rate which became significant after 5 min and declined steadily to 67% of baseline during the one hr observation period (Figure 15).

Neither vagotomy, atropine nor hexamethonium attenuated the immediate hypotensive response produced by WR 194,965-H₃PO₄

(Figure 14). However, propranolol pretreatment enhanced the hypotensive phase produced by WR 194,965-H₃PO₄. The percentage drop in blood pressure was significantly greater at both one (33% of baseline) and 5 (45% of baseline) min after injection when compared to the control WR 194,965-H₃PO₄ response, but had returned to baseline levels 10 min after injection.

None of the 4 pretreatment regimens produced statistically significant alterations in the respiratory response to WR 194,965-H₃PO₄ (Table 19). As seen with the control group the respiratory rate increased to approximately 200% of baseline values within one min after start of injection after pretreatment with propranolol, atropine or hexamethonium. An increase in respiratory rate of 46% was observed in the vagotomized group. The individual variation in respiratory rates makes interpretation of their changes most difficult.

WR 194,965-H₃PO₄ continued to produce a significant decrease in heart rate beginning 5 min after injection in those animals pretreated with atropine, hexamethonium or vagotomy. However, propranolol pretreatment accelerated the onset of this response; a significant decrease in heart rate was observed one min after the WR 194,965-H₃PO₄ injection. Nevertheless, all 4 pretreatment regimens attenuated the steady decline in heart rate produced by WR 194,965-H₃PO₄ during the one hr observation period. This attenuation of the heart rate response after the WR 194,965-H₃PO₄ injection was observed at 40 min in vagotomized dogs, 20 min in propranolol or atropine pretreated animals and 10 min in the beagles pretreated with hexamethonium. The maximum decline in heart rate in the pretreated animals was to 76% of baseline in vagotomized or propranolol pretreated animals, 80% of baseline in atropine pretreated animals and 89% of baseline in the hexamethonium pretreated animals.

d. Discussion:

Previously, we have reported that bolus injections of WR 194,965-H₃PO₄ produced a transient hypotension and tachypnea with reflex tachycardia followed by a long-lasting negative chronotropic effect. However, when WR 194,965-H₃PO₄ was infused slowly over a one hr period, only the long-lasting negative chronotropic activity was observed. In the present study, injection of WR 194,965-H₃PO₄ over a one min period produced a continuous fall in blood pressure which became maximal at the termination of the injection. This continuous fall in blood pressure may explain the absence of the reflex tachycardia and the attenuation of the tachypnea observed following bolus

injections. As in the earlier study, the only cardiorespiratory effect that was not transient was the negative chronotropism produced by WR 194,965-H₃PO₄. Thus the reduction in heart rate is not dosage-rate dependent.

Pharmacological or surgical interruption of the autonomic nervous system produced modifications in the cardiorespiratory responses following WR 194,965-H₃PO₄ injection. The respiratory rate changes due to administration of WR 194,965-H₃PO₄ (two-fold increase in control animals) was reduced to only a 46% increase in animals dosed after bilateral vagotomy. Although this attenuation was not statistically significant, it is consistent with disruption of the afferent reflex responses following bilateral vagotomy. Neither atropine, hexamethonium nor propranolol produced changes in the respiratory response.

Pretreatment with hexamethonium, atropine or bilateral vagotomy had little effect on the hypotensive response produced by the WR 194,965-H₃PO₄ injection. However, pretreatment with propranolol enhanced the magnitude and duration of the hypotensive response produced by WR 194,965-H₃PO₄. This study was not designed to determine the mechanisms for the interaction of propranolol and WR 194,965-H₃PO₄. However, results of a preliminary study with WR 194,965-H₃PO₄ in an isolated canine heart preparation suggest that the drug has a direct negative inotropic action, which could account for the hypotensive response observed following injection of WR 194,965-H₃PO₄ *in vivo*. This negative inotropic action combined with the considerable negative inotropy produced by the administered dose of propranolol could explain the greater reduction in blood pressure produced by WR 194,965-H₃PO₄ following propranolol. The prolonged recovery time is most likely attributable to the magnitude of the hypotensive response, the two drugs' combined negative inotropic activity and to the elimination by beta adrenergic blockade with propranolol of any sympathetic reflex cardiac compensation.

WR 194,965-H₃PO₄ may produce its negative chronotropic action by increasing vagal tone, by decreasing sympathetic tone and/or by decreasing the sinoatrial firing rate directly. The pretreatment regimens were selected for their effectiveness in decreasing sympathetic and/or vagal tone in order to ascertain the relative influence of indirect autonomic mechanisms in the negative chronotropic action of WR 194,965-H₃PO₄. A significant component of the negative chronotropism produced by WR 194,965-H₃PO₄ was considered to be due to a direct action

on sinoatrial fibers as none of the pretreatment regimens prevented WR 194,965·H₃PO₄ from producing a significant decrease in heart rate.

Propranolol pretreatment did attenuate the negative chronotropic action of WR 194,965·H₃PO₄. However, this was considered to be a function of the baseline heart rate, which was reduced following propranolol pretreatment, rather than due to an elimination of a sympatholytic effect of WR 194,965·H₃PO₄. This lack of a WR 194,965·H₃PO₄-induced sympatholytic effect is supported by our previous observation that WR 194,965·H₃PO₄ did not modify the positive chronotropic action of isoproterenol. Although the baseline heart rate was also reduced, the attenuation in the WR 194,965·H₃PO₄ chronotropic response by hexamethonium was considered to be a result of the pretreatment, which in addition to blocking sympathetic tone also blocked parasympathetic tone. This suggests that a vagotonic component may contribute to the negative chronotropism of WR 194,965·H₃PO₄. As anticipated, bilateral vagotomy or atropine pretreatment, which did not affect the baseline heart rate in the pentobarbital-anesthetized dogs, significantly attenuated the negative chronotropism produced by WR 194,965·H₃PO₄.

In conclusion, WR 194,965·H₃PO₄ had prominent cardio-respiratory actions following intravenous injections. The hypotension and tachypnea were transient and/or reflexive responses while the negative chronotropism was relatively long lasting. The negative chronotropism appeared to be a combination response which included a direct action of WR 194,965·H₃PO₄ on sinoatrial fibers and an indirect action mediated via the parasympathetic nervous system.

9. Development of new antimalarial drugs.

a. Background:

The Department of Pharmacology is also charged with the responsibility of writing Notice of Claimed Investigational Exemption for New Drug (IND) submissions. These include planning and designing the experiments, and assembling, evaluating, coordinating and correlating the data required for both the initial submission and all supplementary submissions for each drug. The data must be continuously monitored and evaluated from both in-house and contract sources, as well as proprietary and open literature sources.

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b. Investigational New Drug submissions:

One new IND application was written. It was for
WR 229,870.

Fifteen supplements to IND submissions were written.
Two were for combination drugs and the remainder was for single
drugs.

c. Technical monitoring of contracts necessary for data
generation:

Eleven active contracts were closely guided by the
Department. These ranged from pharmacological areas such as
toxicology and drug metabolism to those of drug formulation
and development of methods to determine blood levels of drugs.

Table 1
Percent of Acetonitrile in Various
Ethylene Dichloride/Hexane (EC/H) Ratios

EC/H 9/1 + A% ^a	EC/H 4/1 + A% ^a	EC/H 7/3 + A% ^a	EC/H 3/2 + A% ^a	EC/H 1/1 + A% ^a
0	0	0	0	-- ^b
0.99	0.99	0.99	0.99	0.99
3.8	3.8	3.8	3.8	3.8
7.4	7.4	7.4	7.4	7.4

^a% acetonitrile added calculated as % of final total volume.

^bNot chromatographed.

Table 2
Column Selectivity with Respect to Varying Acetonitrile Concentrations at Several
Ethylene Dichloride/Hexane (EC/H) Ratios

%A	EC/H Ratio			
	9/1	4/1	7/3	3/2
0	1.666 \pm 0.027 ^a	1.606 \pm 0.014	1.656 \pm 0.015	1.596 \pm 0.015
0.99	1.561 \pm 0.020	1.512 \pm 0.009	1.529 \pm 0.027	1.508 \pm 0.015
3.8	1.421 \pm 0.041	1.330 \pm 0.009	1.342 \pm 0.007	1.270 \pm 0.006
7.4	1.250 \pm 0.018	1.178 \pm 0.007	1.137 \pm 0.004	1.126 \pm 0.008
^a Mean \pm standard deviation of selectivity at various EC/H ratios with respect to the acetonitrile concentration.				
^b The 1/1 ratio of ethylene dichloride/hexane with 0% acetonitrile was omitted because of extremely long retention volumes needed to elute the candidate antimalarial and its internal standard.				
				1.438 \pm 0.014
				1.163 \pm 0.018
				1.062 \pm 0.007

Table 3
Mean and Standard Deviation at Several Combinations
of Major and Minor Components^a

EC/H/%A 9/1/0%	EC/H/%A 3/2/3.8%	EC/H/%A 3/2/0%	EC/H/%A 4/1/3.8%
2.738 \pm 0.61	2.720 \pm .049	2.776 \pm .071	2.952 \pm .084
1.828 \pm .053	1.763 \pm .076	1.745 \pm .051	1.955 \pm .053
1.361 \pm .016	1.342 \pm .042	1.40 \pm .063	1.482 \pm .076
.702 \pm .015	.678 \pm .019	.698 \pm .032	.741 \pm .019
.350 \pm .021	.314 \pm .014	.366 \pm .023	.365 \pm .015
.196 \pm .014	.171 \pm .014	.186 \pm .015	.202 \pm .011

^aEC is ethylene dichloride, H is hexane and A is acetonitrile.

Table 4

Flow Chart for General Assay Procedure for WR 142,490

- A-1. Blood sample (volumetric or weighed aliquot).
- A-2. Add internal standard (50 μ l WR 184,806 solution) and mix on vortex.
- A-3. Add buffer (5 ml, pH 7.4 phosphate buffer) and mix on vortex.
- A-4. Add extractant (10 ml ethyl acetate) and mix on vortex.
- A-5. Agitate for 1/2 hour on shaker.
- A-6. Centrifuge (2000 rpm for 10 min) to separate.

Blood

- B-1. Add second 10 ml aliquot of ethyl acetate to blood
- B-2. Shake for 1/2 hour
- B-3. Centrifuge to separate

Blood

Supernatant 2
(add to Supernatant 1)

- C-1. Add third 10 ml aliquot of ethyl acetate to blood
- C-2. Shake for 1/2 hr
- C-3. Centrifuge to separate

Blood
(discard)

Supernatant 3
(add to Supernatant 1)

Supernatant 1 + Supernatant 2 + Supernatant 3

- D-1. Combine, dry to form solid residue
- D-2. Re-dissolve in 500 μ l mobile phase
- D-3. Chromatograph 50 μ l aliquots
- D-4. Quantitate chromatograms in μ g/gram blood sample

Table 5

Recovery of WR 142,490 from Spiked Blood Samples^a

<u>Blood Samples Maintained at Room Temperature</u>			
Number of Determinations	Concentration of WR 142,490 Determined by This Method $\bar{X} \pm S_m$	Actual Concentration of the Prepared Samples	% Error
3	2.427 \pm 0.080 $\mu\text{g/g}$	2.440 $\mu\text{g/g}$	-0.53%
4	0.828 \pm 0.016 "	0.840 "	-1.43%
4	0.397 \pm 0.004 "	0.422 "	-5.92%
4	0.064 \pm 0.001 "	0.067 "	-4.48%
<u>Blood Samples Frozen and Thawed to Room Temperature Before Analysis</u>			
Number of Determinations	Concentration of WR 142,490 Determined by This Method $\bar{X} \pm S_m$	Actual Concentration of the Prepared Samples	% Error
4	2.334 \pm 0.009 $\mu\text{g/g}$	2.440 $\mu\text{g/g}$	-4.34%
5	0.777 \pm 0.006 "	0.840 "	-7.50%
4	0.405 \pm 0.005 "	0.422 "	-4.03%
4	0.072 \pm 0.003 "	0.067 "	+7.46%

^aThe replicates at a given concentration were obtained as aliquots of a given larger spiked pool.

Table 6
Recovery of WR 142,490 from Spiked Blood Samples^a

<u>Experiment 1</u>				
Number of Determinations	Concentration of WR 142,490 Determined by This Method $\bar{X} \pm S_m$	Actual Concentration of the Prepared Samples	% Error	
3	1.966 \pm 0.024 $\mu\text{g/ml}$	2.020 $\mu\text{g/ml}$	-2.67%	
3	1.554 \pm 0.060 "	1.515 "	+2.57%	
3	0.969 \pm 0.027 "	1.010 "	-4.06%	
3	0.505 \pm 0.013 "	0.505 "	0.00%	
3	0.146 \pm 0.007 "	0.150 "	-2.67%	
3	0.061 \pm 0.002 "	0.040 "	+22.00%	
<hr/>				
<u>Experiment 2</u>				
4	1.833 \pm 0.039 $\mu\text{g/ml}$	1.883 $\mu\text{g/ml}$	-2.66%	
4	1.389 \pm 0.014 "	1.412 "	-1.63%	
4	0.884 \pm 0.016 "	0.941 "	-6.06%	
4	0.442 \pm 0.004 "	0.471 "	-6.16%	
4	0.137 \pm 0.003 "	0.141 "	-2.84%	
4	0.052 \pm 0.003 "	0.047 "	+10.64%	

^aThe replicates at a given concentration were spiked individually rather than being aliquots of a larger pool as in Table 5.

Table 7
Excretion of Total Radioactivity and Unchanged Drug after
Oral (PO) or Intraperitoneal (IP) Administration
of Mefloquine-¹⁴C (10 mg/kg) to Mice^a

Time (hr)	Urine		Feces	
	PO	IP	PO	IP
0-12	2.65 (44.3)	3.66 (39.1)	13.10 (61.7)	12.67 (58.8)
12-24	2.47 (23.1)	2.87 (22.2)	13.13 (50.4)	9.54 (51.0)
24-36	4.72 (16.6)	3.20 (22.6)	9.14 (32.5)	12.58 (43.5)
36-48	3.15 (10.2)	3.12 (15.0)	7.23 (21.0)	6.40 (31.8)
48-60	2.34 (10.8)	2.98 (12.1)	12.68 (27.9)	9.52 (24.6)
60-72	1.47 (8.6)	2.31 (4.5)	6.54 (30.6)	6.13 (24.1)
72-84	1.17 (2.9)	1.55 (7.6)	3.28 (28.3)	4.82 (23.2)
84-96	.92 (8.1)	1.15 (6.9)	2.44 (22.9)	3.37 (12.2)
96-108	.52 (6.1)	.45 (2.8)	1.60 (14.3)	1.11 (11.1)
108-120	.27 (--) ^b	.38 (--) ^b	1.53 (--) ^b	1.50 (--) ^b
120-132	.20 (--)	.21 (--)	.44 (--)	.34 (--)
132-144	.12 (--)	.15 (--)	.79 (--)	.47 (--)
144-168	.08 (--)	.13 (--)	.32 (--)	.60 (--)
168-192	.04 (--)	.06 (--)	.19 (--)	.30 (--)
192-216	.03 (--)	.04 (--)	.16 (--)	.17 (--)
216-240	.02 (--)	.02 (--)	.09 (--)	.09 (--)
(%) Total Excretion	20.17	22.28	72.66	69.61

^aSix mice were dosed per administration route, with determinations made on the appropriate pooled excreta. Values in parentheses are the percentages of each excreta sample identified as parent drug. The numbers to the left of the parentheses represent the percent of the dose excreted during each time period.

^bValues were too low to measure.

Table 8

Concentration of ^{14}C -Drug Equivalents in the Plasma and RBC of the Mouse after a Single Oral Dose of Mefloquine- ^{14}C (8.4 mg/kg)

Time	Drug Equivalents ^a			
	Plasma		Red Blood Cells	
	Total	Mefloquine ^b	Total	Mefloquine ^b
hr	$\mu\text{g/ml}$			
4	0.96	0.29	1.69	0.98
8	1.17	0.19	1.72	1.00
24	1.55	0.12	1.59	0.70
48	0.95	0.04	0.78	0.21
72	0.57	0.02	0.46	0.10
96	0.48	<0.01	0.32	<0.01

^aSix mice per time period with a single determination from their pooled blood.

^bArea of radioactivity on TLC plates of R_f corresponding to mefloquine.

Table 9

Tissue Distribution of Total Radioactivity and Unchanged Drug^a

Tissue	Percentage of Dose at	
	24 hr	48 hr
Submaxillary Salivary Glands	0.67 (73)	0.21 (66)
Heart	0.41 (47)	0.23 (28)
Lungs	4.91 (68)	2.29 (72)
Spleen	0.56 (59)	0.19 (84)
Kidneys	1.91 (71)	0.98 (56)
Liver	7.66 (72)	6.78 (62)
Gall bladder + Bile	0.11 (19)	0.15 (12)
Stomach + Contents	2.66 (65)	0.82 (63)
Small Intestine + Contents	10.18 (71)	5.63 (54)
Cecum + Contents	4.03 (29)	2.47 (25)
Large Intestine + Contents	5.35 (38)	3.36 (30)
Plasma	0.16 (8)	0.18 (4)
Red Blood Cells	0.14 (44)	0.13 (27)
Carcasses	29.21 (79)	14.60 (64)
Urine ^b	8.77 (34)	16.42 (22)
Feces ^b	15.27 (53)	37.52 (37)
Total Recovery (%)	91.90	91.96

^aMefloquine-¹⁴C was administered orally, 10 mg/kg. Values in parentheses are percentage of total radioactivity represented by unchanged drug. Four mice were used per time period, with determinations made on their pooled tissues.

^bCumulative samples from dosing to kill-times.

Table 10
Percent of WR 177,602-¹⁴C Derived Radioactivity Recovered in the
Urine and Feces of Mice after Oral Administration of the Drug^a

Time (hr)	Percent ¹⁴ C Recovered ^b	
	Urine	Feces
0-12	7.96	12.47
12-24	0.78	11.35
24-36	8.52	26.31
36-48	2.28	8.06
48-60	1.81	6.01
60-72	0.85	2.35
72-96	0.71	2.11
96-144	0.41	0.82
144-168	<u>0.06</u>	<u>0.23</u>
Cumulative Recovery	23.38	69.71

^aWR 177,602-¹⁴C was administered 10 mg/kg orally.

^bAverage of two cages of 4 mice each.

Table 11

Percent of Parent Drug in Plasma per Time Period

Time (hr)	% Unchanged Drug ^a
12	81.46
24	81.48
36	77.34
48	81.86
60	70.00
72	55.88
96	37.50

^aPercentage of total plasma radioactivity represented by material of R_f corresponding to WR 177,602.

Table 12
Tissue Distribution and Concentration of WR 177,602-Derived Radioactivity^a

Tissue	% Administered Dose ^b		
	2 hr	6 hr	24 hr
Brain	0.53 (2.66)	0.83 (4.05)	0.36 (1.83)
Eyes	0.34 (11.81)	0.84 (25.56)	0.51 (16.57)
Submaxillary Salivary Glands	0.63 (12.78)	0.65 (12.47)	0.25 (4.81)
Heart	0.55 (8.75)	0.62 (9.36)	1.08 (15.75)
Lungs	4.18 (54.85)	5.72 (82.49)	3.03 (40.59)
Liver	13.46 (26.00)	13.57 (27.32)	7.27 (13.13)
Gall Bladder + Bile	0.34 (38.67)	0.52 (65.68)	0.24 (55.31)
Spleen	0.86 (18.21)	0.94 (20.26)	0.27 (6.46)
Kidneys	4.06 (27.77)	3.77 (26.67)	1.73 (11.81)
Adrenal Glands	<0.01 (0)	0.06 (9.05)	<0.01 (0)
G.I. Tract + Contents	41.63 (40.34)	28.54 (26.14)	28.03 (21.52)
Abdominal Fat	c (2.66)	c (3.93)	c (1.97)
Skeletal Muscle	c (4.66)	c (5.29)	c (2.81)
Carcasses	43.03 (d)	43.67 (d)	21.50 (d)

^aWR 177,602-¹⁴C was administered orally, 10 mg/kg. Four mice were used per time period.

^b μ g ¹⁴C-drug equivalent/g tissue are listed in parentheses.

^cOnly a small portion of the tissue was taken out.

^d μ g ¹⁴C-drug equivalent/g tissue was not calculated.

Table 13
Partition Coefficients of WR 177,602^a

Organic Solvents	Kp
Ethylacetate	140.04 \pm 4.13
Diethylether	100.46 \pm 9.15
N-Butanol	96.44 \pm 23.59
Chloroform	72.94 \pm 3.39
Benzene	40.98 \pm 0.13
N-Heptane	1.37 \pm 0.03

^aData represent means \pm S.E.M. of three determinations. Kp is expressed as the ratio of the concentration of drug in the organic phase to that in the aqueous phase (pH 7.4 phosphate buffer).

Table 14

Percent of WR 172,435- ^{14}C Derived Radioactivity Recovered in the Urine and Feces of Mice After Oral Administration of the Drug^a

Time (hr)	Percent ^{14}C Recovered ^b	
	Urine	Feces
0-12	0.14	58.00
12-24	0.05	4.40
24-36	0.09	4.00
36-48	0.04	1.40
48-60	0.07	1.50
60-72	0.03	0.80
72-84	0.02	0.90
84-96	0.04	0.40
96-108	0.02	0.90
108-120	0.08	0.50
120-144	0.06	1.30
144-168	0.04	1.20
168-192	0.04	1.10
Total Recovery (%) ^c	0.72	76.40

^aWR 172,435- ^{14}C was administered 20 mg/kg orally.

^bAverage of two cages of 4 mice each.

^cFinal total percent ^{14}C recovery including residual carcasses (18.82%) was 95.94%.

Table 15
Metabolic Profile of WR 172,435-CH₃SO₃H in Mice Fecal Extracts
Before and After Hydrolysis

Hydrolysis System	Rf ^a	
	Parent	Metabolite
Before Hydrolysis	0.70	0.43
Buffer	0.65	0.43
β-glucuronidase	0.66	0.43
β-glucuronidase/ aryl sulfatase	0.61	0.43
0.2 N HCl	0.70	0.46

^aTLC solvent system - BuOH:HOAc:H₂O (10:1:1).

Table 16

WR 172,435-CH₃SO₃H Equivalents in the Plasma and Red Blood Cells after Oral Administration of 20 mg/kg of the Drug in Mice

Hour	$\mu\text{g/ml}$	
	Plasma	RBC
0.5	0.54	1.26
1.0	1.09	1.48
1.5	2.19	0.77
4.0	1.09	0.74
6.0	0.44	0.56
24.0	0.43	0.49
72.0	0.12	0.15
120.0	0.11	0.08

Table 17
Tissue Distribution of Total Radioactivity and Unchanged Drug^a

Tissue	Percentage of Dose at					
	2 hr	6 hr	24 hr	48 hr	72 hr	120 hr
Brain	0.01 (b)	0.03 (b)	0.04 (b)	0.06 (b)	0.05 (b)	0.05 (86)
Eyes	0.02 (b)	0.03 (b)	0.06 (b)	0.07 (b)	0.07 (b)	0.08 (76)
Submaxillary Salivary Glands	0.04 (57)	0.14 (78)	0.32 (81)	0.22 (86)	0.40 (85)	0.19 (86)
Heart	0.12 (73)	0.38 (94)	0.15 (94)	0.13 (94)	0.10 (87)	0.08 (75)
Lungs	0.26 (92)	0.42 (93)	0.59 (b)	0.50 (89)	0.38 (92)	0.28 (74)
Gall Bladder	0.17 (9)	0.07 (20)	0.10 (84)	0.12 (18)	0.13 (25)	0.01 (49)
Liver	6.01 (96)	6.26 (83)	4.29 (80)	3.05 (85)	3.36 (81)	2.44 (70)
Spleen	0.13 (93)	0.16 (94)	0.18 (91)	0.12 (77)	0.13 (96)	0.03 (90)
GI Tract	65.66 (68)	14.54 (66)	7.03 (33)	5.19 (36)	2.29 (73)	2.46 (98)
Adrenal Glands	0.01 (b)	0.02 (b)	0.04 (b)	0.05 (95)	0.39 (b)	0.05 (86)
Kidneys	0.30 (72)	0.68 (83)	1.26 (92)	1.72 (97)	0.84 (70)	0.65 (74)
Abdominal Fat	c (80)	c (88)	c (92)	c (89)	c (95)	c (98)
Skeletal Muscle	c (95)	c (4)	c (92)	c (85)	c (90)	c (71)
Carcasses	3.75 (d)	47.31 (d)	48.80 (d)	38.98 (d)	18.14 (d)	18.04 (d)

Table 17 - Continued

^aWR 172,435-¹⁴C was administered orally, 20 mg/kg. Values in parentheses are percentages of total radioactivity in the sample represented by unchanged drug. Four mice were used per time period.

^bInsufficient sample and/or radioactivity to perform analysis.

^cOnly a small portion of the tissue was taken out and the amount of total radioactivity determined was included with that of the carcasses.

^dNot determined.

Table 18
Tissue Concentration of WR 172,435 Equivalents

Tissue	$\mu\text{g/g}$				
	2 hr	6 hr	24 hr	48 hr	72 hr
Brain	3.40	10.76	12.96	19.53	16.72
Eyes	44.48	57.44	98.88	135.93	134.40
Submaxillary Salivary Glands	28.08	81.44	209.64	158.40	241.48
Heart	99.96	300.76	130.32	114.03	90.84
Lungs	220.32	359.76	539.44	408.42	262.72
Gall Bladder	1139.63	642.60	1083.40	631.33	159.44
Liver	679.60	548.80	362.08	302.61	243.80
Spleen	231.52	268.72	302.32	190.20	179.72
GI Tract	4418.08	884.76	365.16	296.91	94.68
Adrenal Glands	242.00	270.28	523.44	502.32	420.08
Kidneys	100.12	211.32	409.52	454.65	241.60
Abdominal Fat	27.52	41.96	166.56	240.57	247.24
Skeletal Muscle	23.40	42.20	73.52	83.61	90.04

Table 19

Percentage Change in Baseline Respiratory Rate of the Beagle Dog
Following Various Pretreatment Regimens^a

Group	Pre WR 194,965 Baseline	Time in Minutes after Start of WR 194,965-H ₃ P04 Injection						
		1	5	10	20	30	40	50
Control (No Pretreatment)	8.2 ^b + 1.3	208 ^c + 36	175 + 30	134 + 28	141 + 26	135 + 24	131 + 26	139 + 25
Vagotomy	9.4 + 3.4	146 + 33	66 + 3	73 + 3	79 + 4	76 + 3	75 + 2	70 + 8
Atropine	12.0 + 2.8	246 + 30	169 + 36	134 + 14	115 + 12	93 + 11	94 + 13	84 + 12
Hexamethonium	14.6 + 2.9	192 + 23	166 + 19	115 + 6	99 + 3	96 + 9	93 + 9	88 + 6
Propranolol	21.2 + 6.4	164 + 41	235 + 59	186 + 36	142 + 36	126 + 34	118 + 36	119 + 37

^aDoses of WR 194,965-H₃P04 (15 mg/kg), atropine sulfate (1 mg/kg), hexamethonium-HCl (10 mg/kg) refer to the base. The dose of propranolol-HCl (2 mg/kg) was based on the salt.

^bValues represent mean \pm SEM of actual baseline respiratory rate for 5 animals.

^cValues represent mean \pm SEM percent of baseline values for 5 animals.

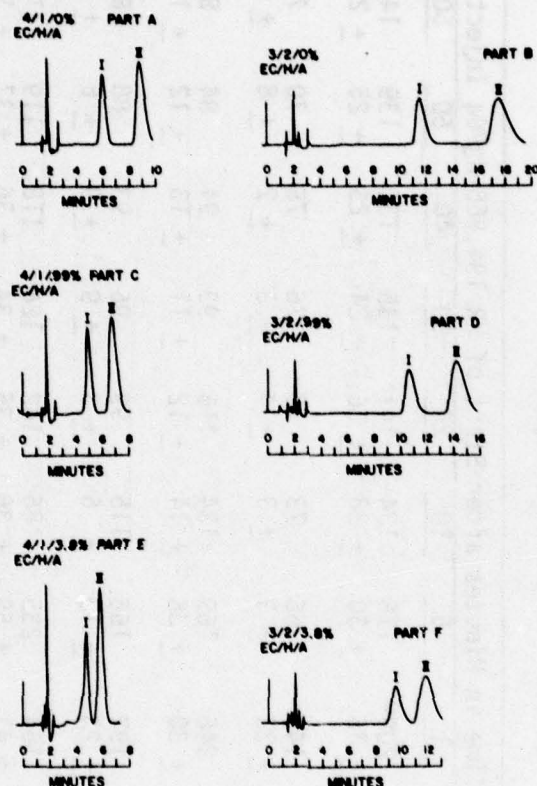


Figure 1. Parts A and B. The effects of solvent strength on column retention times for the candidate antimalarial WR 180,409·H₃PO₄, and its internal standard, WR 184,806·H₃PO₄. EC = Ethylene Dichloride; H = Hexane; A = Acetonitrile; Peak I = WR 184,806·H₃PO₄; Peak II = WR 180,409·H₃PO₄. Parts C, D, E and F. The effects of solvent strength on column retention times with respect to various acetonitrile concentrations on column selectivity for the candidate antimalarial WR 180,409·H₃PO₄, and its internal standard, WR 184,806·H₃PO₄. Peak I = WR 184,806·H₃PO₄; Peak II = WR 180,409·H₃PO₄.

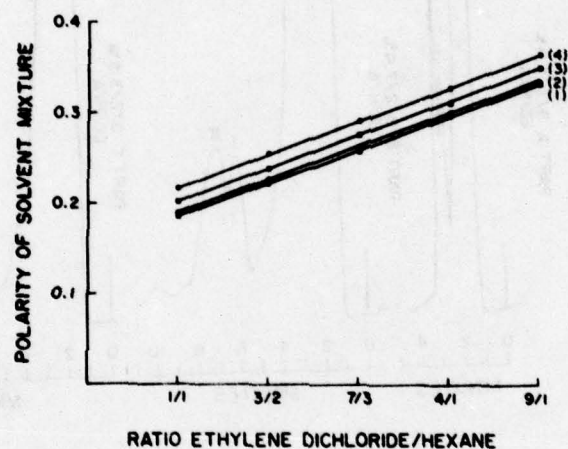


Figure 2. The polarity of the solvent mixture with respect to the ethylene dichloride/hexane ratio at varying concentrations of acetonitrile (A). (1) Acetonitrile = 0%; (2) Acetonitrile = 0.99%; (3) Acetonitrile = 3.8%; (4) Acetonitrile = 7.4%.

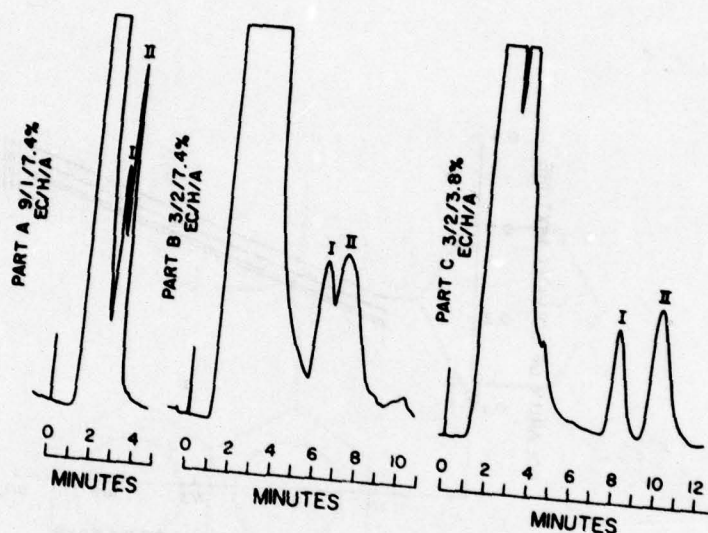


Figure 3. A blood extract containing the candidate antimalarial WR 180,409·H₃PO₄, and its internal standard, WR 184,806·H₃PO₄. Part A and Part B demonstrate the ability to isolate the antimalarial (peak II) and its internal standard (peak I) from significant interference patterns, while simultaneous alterations of minor component (Part C) will effect separation of the candidate antimalarial from the internal standard. A = Acetonitrile; EC = Ethylene Dichloride; H = Hexane; Peak I - WR 184,806·H₃PO₄; Peak II = WR 180,409·H₃PO₄.

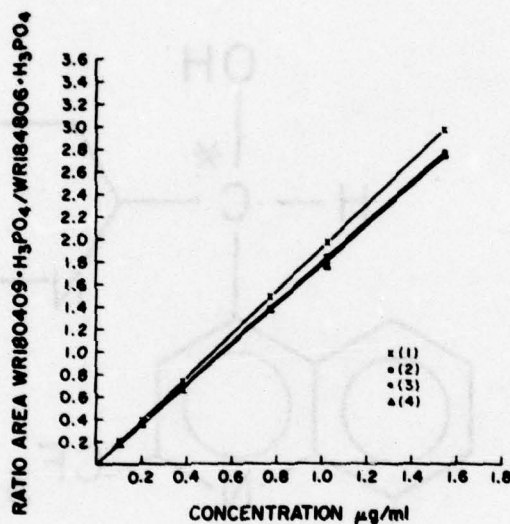


Figure 4. Calibration curves of the ratio of the area for the chromatogram of the antimalarial WR 180,409-H₃PO₄ (peak II) with respect to the area of the chromatogram of its internal standard (peak I) at several concentrations (μg/ml) of the antimalarial WR 180,409-H₃PO₄. Four calibration curves (1-4) were established at several combinations of major and minor components.

(1) 4 1 3.8% (2) 3 2 0% (3) 3 2 3.8% (4) 9 1 0%
 EC H A EC H A EC H A EC H A

EC = Ethylene Dichloride; A = Acetonitrile; H = Hexane.

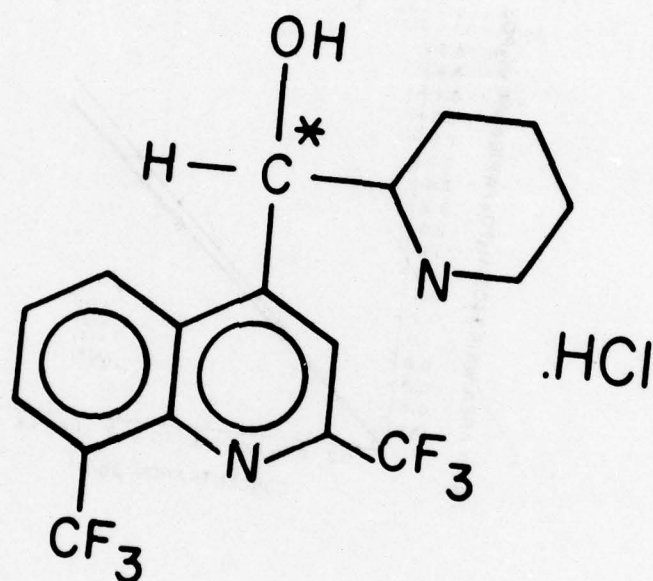


Figure 5. WR 177,602·HCl. Threo- α -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride. The asterisk denotes the position of the radioactive carbon.

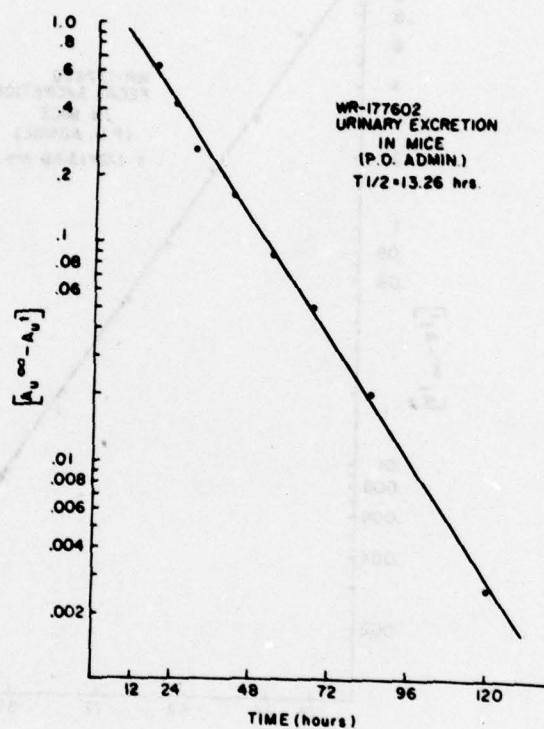


Figure 6. Linear regression analysis of residual radioactivity versus time plot for urine after oral administration of 10 mg/kg WR 177,602-HCl in mice.

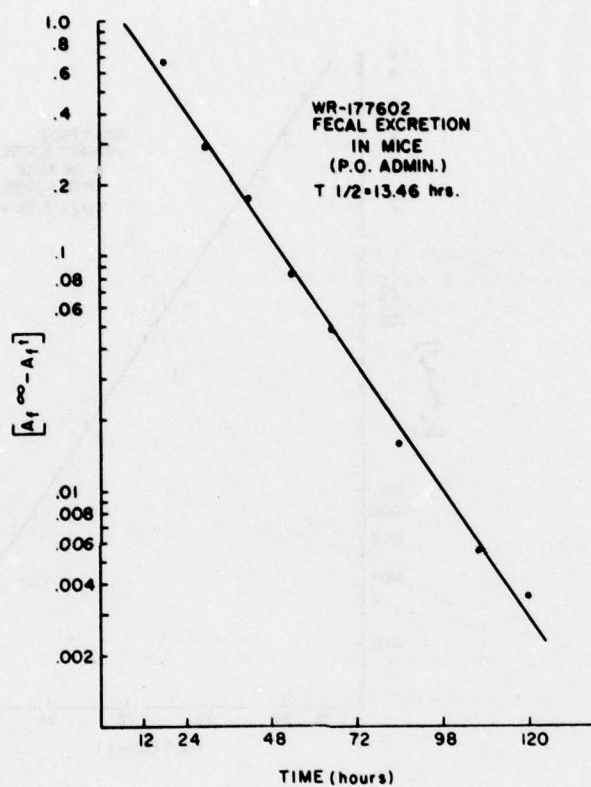


Figure 7. Linear regression analysis of residual radioactivity versus time plot for feces after oral administration of 10 mg/kg WR 177,602·HCl in mice.

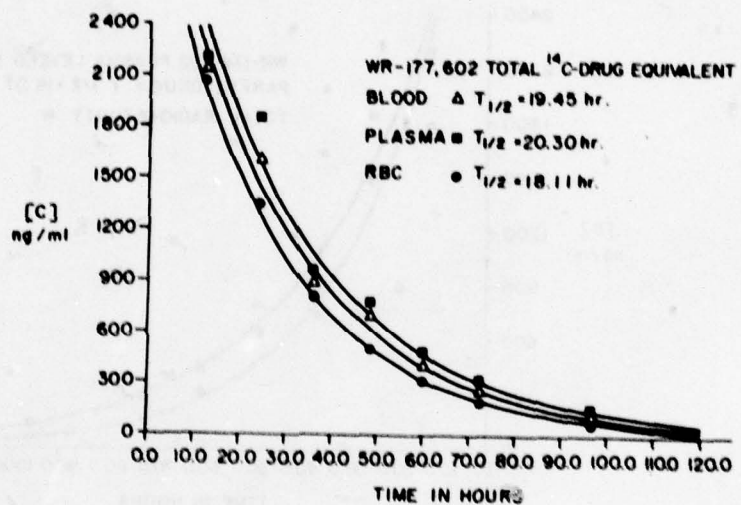


Figure 8. WR 177,602 total ^{14}C -drug equivalents in blood, plasma and RBC.

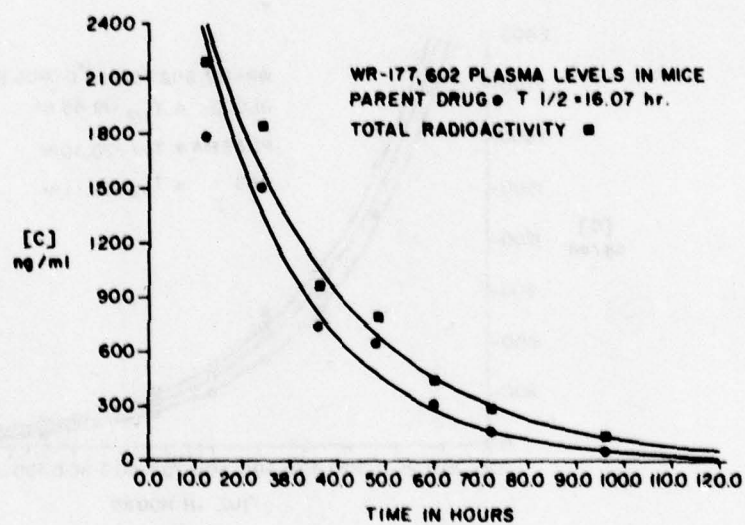
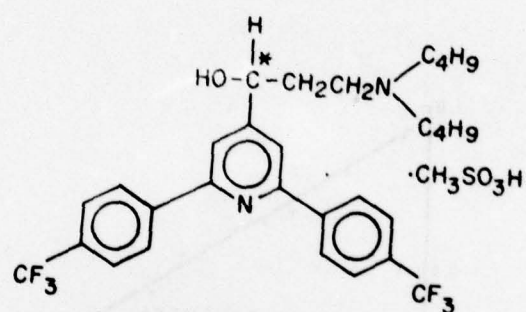


Figure 9. WR 177,602 plasma levels in mice.



WR 172, 435 · CH₃SO₃H

3-Di-n-butylamino-1-[2,6-bis(4-trifluoromethylphenyl)-4-pyridyl]-propanol methanesulfonate

Figure 10. WR 172,435·CH₃SO₃H. 3-di-n-butylamino-1-[2,6-bis(4-trifluoromethylphenyl)-4-pyridyl]-propanol methanesulfonate. The asterisk denotes the position of the radioactive carbon.

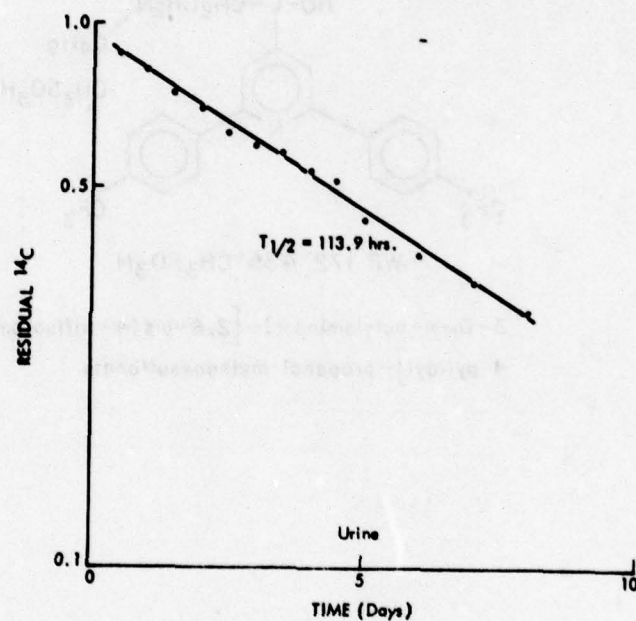


Figure 11. Linear regression analysis of residual radioactivity versus time plot for urine after oral administration of 20 mg/kg WR 172,435- $\text{CH}_3\text{SO}_3\text{H}$ in mice.

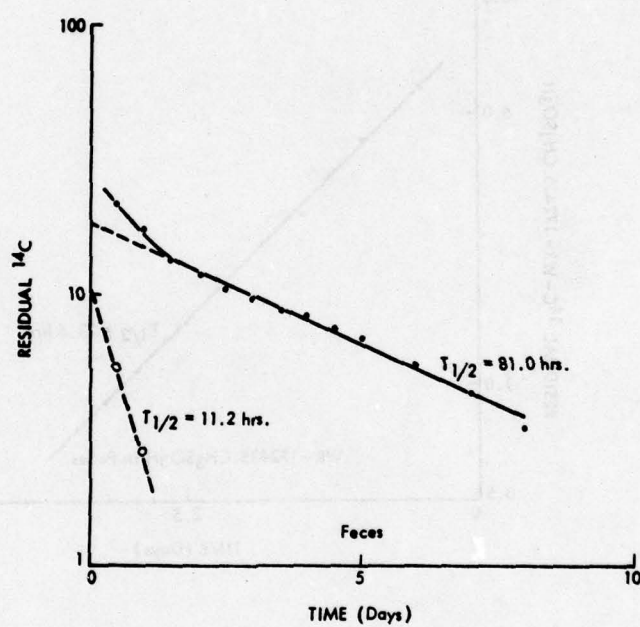


Figure 12. Linear regression analysis of residual radioactivity versus time plot for feces after oral administration of 20 mg/kg WR 172,435- $\text{CH}_3\text{SO}_3\text{H}$ in mice.

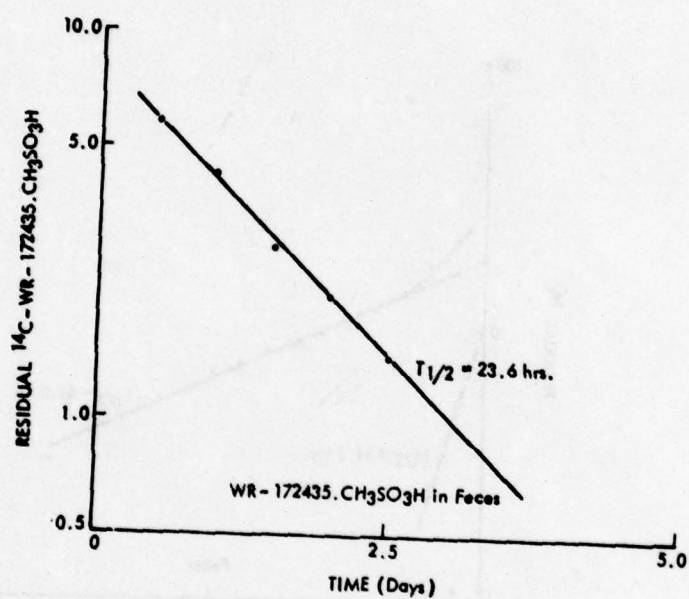


Figure 13. Linear regression analysis of residual WR 172,435 versus time plot for feces after oral administration of 20 mg/kg WR 172,435·CH₃SO₃H in mice.

MEAN ARTERIAL PRESSURE CHANGES PRODUCED BY
15mg/Kg WR 194,965-H₃PO₄ FOLLOWING VARIOUS PRETREATMENTS

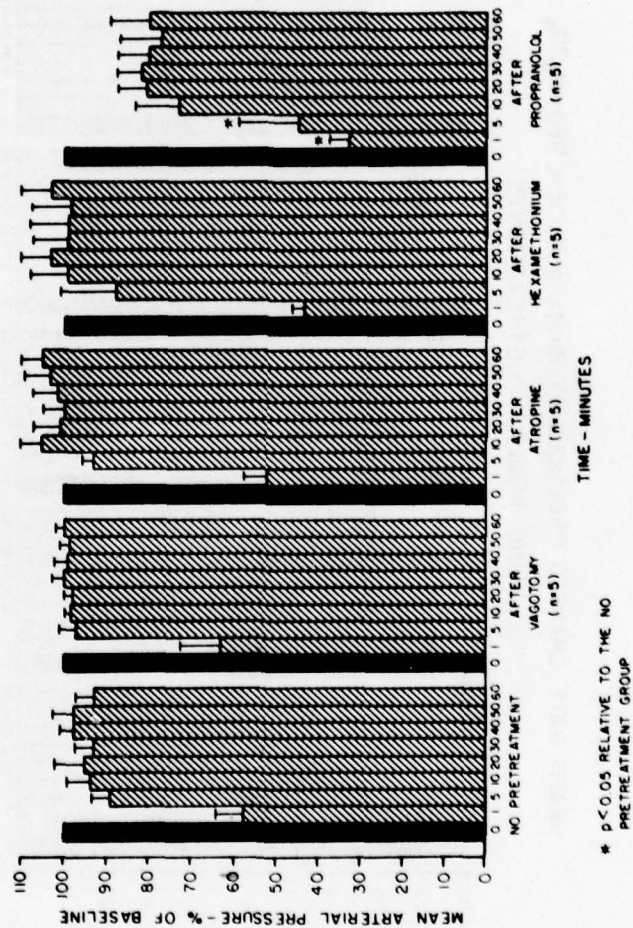


Figure 14.

HEART RATE CHANGES PRODUCED BY 15mg/Kg WR 194, 965-H₃PO₄
FOLLOWING VARIOUS PRETREATMENTS

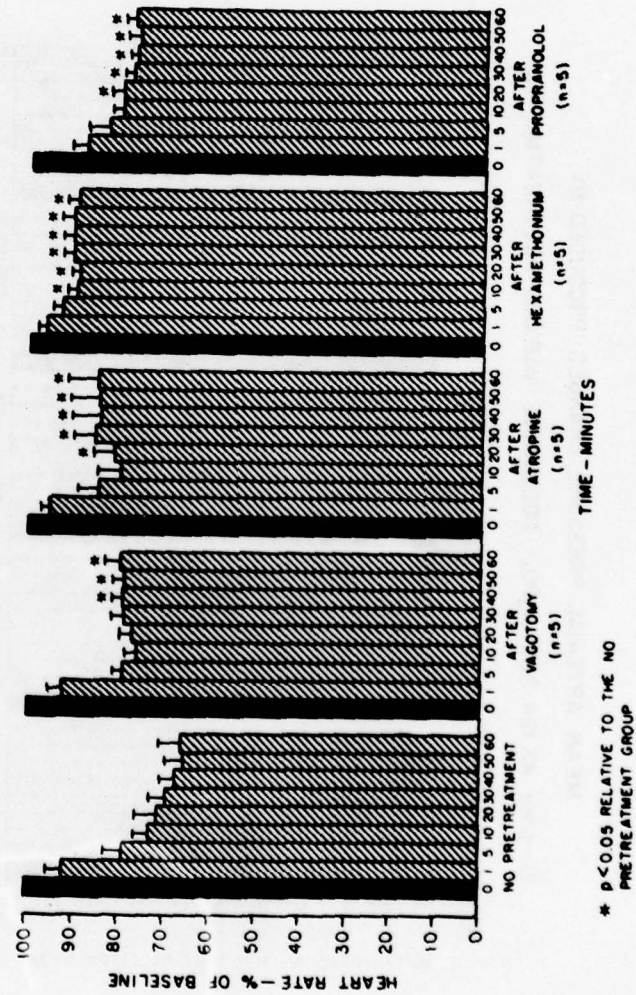


Figure 15.

Project 3M162770A803 MALARIA PROPHYLAXIS

Work Unit 087 Determination of pharmacological effects of anti-malarial drugs

Literature Cited.

References:

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2. Korte, D.W., Jr., Heiffer, M.H., Kintner, L.D., and Lee, C.-C.: Comparative acute and subacute toxicities of erythro- α -(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol HCl, WR-142490-HCl (mefloquine-HCl), and its threo diastereomer, WR-177602-HCl, in dogs. Fed. Proc. 37:248, 1978.
3. Desjardins, R.E., Haynes, J.D., Chular, J.D., and Canfield, C.J.: Quantitative assessment of antimalarial activity by an automated in vitro system. Fed. Proc. 37:379, 1978.
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5. Korte, D.W., Jr., Herman, A., Neidig, M.H., Jr., and Heiffer, M.H.: Cardiovascular activity of the candidate anti-malarial drug 4-(t-butyl)-2-(t-butylaminomethyl)-6-(4-chlorophenyl)phenol phosphate (WR 194,965-H3P04) in the dog. The Pharmacologist 20:253, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION ^a		2. DATE OF SUMMARY ^a		REPORT CONTROL SYMBOL DD-DR&S(AR)35		
					DA OC 6446		78 10 01				
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ECTY ^b	6. WORK SECURITY ^c	7. REGRADING ^d	8a. DRGPR INVTN ^e	9a. SPECIFIC DATA CONTRACTOR ACCESS		9b. LEVEL OF SUP A. WORK UNIT			
77 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO					
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER			
a. PRIMARY		62770A		3M162770A803		00		088			
b. CONTRIBUTING											
c. CONTRIBUTING		CARDS 114 F									
11. TITLE (Precede with Security Classification Code)											
U) Biochemical Research on Antimalarials											
12. SCIENTIFIC AND TECHNOLOGICAL AREA ^a											
002300 Biochemistry 012900 Physiology											
13. START DATE		14. ESTIMATES COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD					
76 07		CONT		DA		C. In-House					
17. CONTRACT/GRANT											
a. DATES/EFFECTIVE:		EXPIRATION:		18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS		b. FUNDS (in thousands)			
b. NUMBER ^a		NA		FISCAL YEAR		78		8		565	
c. TYPE:		d. AMOUNT:		FUNDING YEAR		79		8		429	
e. KIND OF AWARD:		f. CUM. AMT.									
19. RESPONSIBLE S&T ORGANIZATION				20. PERFORMING ORGANIZATION							
NAME ^a Walter Reed Army Institute of Research				NAME ^a Walter Reed Army Institute of Research							
ADDRESS ^a Washington, D.C. 20012				Division of Biochemistry							
				ADDRESS ^a Washington, D.C. 20012							
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Precede with U.S. Academy Profession)							
NAME: RAPMUND, GARRISON, COL, MC				NAME ^a DOCTOR, B.P. Ph.D							
TELEPHONE: (202) 576-3551				TELEPHONE: (202) 576-3001							
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:							
Foreign Intelligence not considered				ASSOCIATE INVESTIGATORS							
				NAME: SLEEMAN, H. KENNETH Ph.D							
				NAME: HANSEN, BRIAN, D. Ph.D							
22. REVISIONS (Precede with Security Classification Code)											
(U) Antimalarials (U) Pharmacokinetics (U) Drug Intolerance (U) Analytical Chemistry											
23. (U) The technical objectives include (1) The determination of biochemical indices that reliably predict drug intolerance in man. (2) The evaluation of effects of parasites and drugs on biochemical mechanisms as indices of prophylaxis and treatment. (3) The gas chromatographic - coupled mass spectrometric analysis of drugs and drug metabolites. (4) Analytical support for special and collaborative projects. The prediction of drug intolerance, the effects of drugs and infection on biochemical mechanisms and the understanding of drug and drug metabolite structure are relevant to the Army's malaria and tropical disease program.											
24. (U) Tissues and fluids from animals will be analyzed to determine metabolic processes affected by parasitic diseases and therapeutic and prophylactic drugs. Metabolic products, enzymes, isoenzymes, nucleic acids, lipids, and proteins will be the primary focus. Immunochemical, spectrophotometric, mass spectrometric, and chromatographic methods will be developed and utilized to analyze biological specimens for compounds of interest.											
25. (U) 77 10 - 78 09 Studies were continued on the effects of antimalarials on metabolic and physiological parameters. The 4-quinolinemethanols were found to depress thyroid gland function. These effects were not seen with the phenanthrenemethanols or chloroquine. The study of the effects of the antimalarials on lipid metabolism was initiated. The membrane transport and utilization of purine bases and nucleosides by normal and infected erythrocytes and free malaria parasites have been determined and the effects of antimalarial drugs on these parameters investigated. The analysis of drugs and drug metabolites by mass spectroscopy was continued. For technical report see WRAR Annual Progress Report 1 Oct 77 to 30 Sept 78.											

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1573

Project 3M16770A803 MALARIA PROPHYLAXIS

Work Unit 088 Biochemical Research in Antimalarials

Investigators

Principal: Bhupendra P. Doctor, Ph.D

Associates: SP/4 Gregory E. Burgess; Kevin J. Crossen, M.S.; Seymour Garson, Ph.D Brian D. Hansen, Ph.D Leo Kazyak, B.S.; PFC Gregory Miller; SFC Evelyn Moore; H. Kenneth Sleeman, Ph.D; Thomas D. Wieland, B.S.

The objective of this work unit are to study biochemistry of malaria, and the other parasitic diseases, biochemical effects of antimalarials, the biochemistry of the host-parasite interactions in the presence of antimalarial drugs, and the speciation of biomolecules by mass spectrometry. Studies on Trypanosoma rhodesiense metabolism are reported.

1. The membrane transport of purine and nucleosides by the normal rat erythrocyte, the Plasmodium berghei-infected erythrocyte, and the free parasite.
 2. Biochemical effects of selected antimalarials on thyroid function in the rat.
 3. Drug metabolism in experimental infection of Plasmodium berghei or Trypanosoma rhodesiense.
 4. Lipid metabolism in malaria: The identification and quantitation of fatty acids.
 5. Gluconeogenesis in experimental trypanosomiasis.
 6. Phthalate plasticizers in biological fluids.
 7. Study of drug metabolites by mass spectrometry:
(a) terbutaline (b) methaqualone.
1. The membrane of purine bases and nucleosides by the normal rat erythrocyte, the Plasmodium berghei-infected erythrocyte and the free parasite.

Numerous investigators have demonstrated that the species of plasmodium infecting higher vertebrates are not capable of de novo purine synthesis, although they do synthesize pyrimidines (Bungener and Nielsen, 1968; Van Dyke,

1977; Polet and Barr, 1968; Walsh and Sherman, 1968). Therefore purines are presumably supplied to the parasite through the host erythrocyte and inhibition of purine uptake may provide an effective treatment for the disease. Once these mechanisms are more completely understood key points of chemotherapeutic attack might be suggested. Of particular importance are the food acquiring mechanism, in particular the membrane transport of the purine ring. The purpose of the following study was to characterize the purine base and nucleoside transport systems in the normal erythrocyte, the Plasmodium berghei-infected erythrocyte and the "free" malarial parasite (isolated by saponin lysis; Van Dyke et. al., 1977). All studies were conducted using the rodent model (Sprague-Dawley male retired breeder rats).

The uptake velocity of two purine bases (adenine and hypoxanthine) and two purine nucleosides (adenosine and inosine) suggested to be of nutritional importance to the malarial parasite (Van Dyke, 1977) were measured and plotted as a function of increasing substrate concentration in the normal erythrocyte, Plasmodium berghei-infected erythrocyte and free parasite. In all cases, a combination of mediated uptake (facilitated diffusion and active transport) and simple diffusion were demonstrated, with the rate of absorption being nonlinear at low substrate concentration (mediation) and linear at high substrate concentration (diffusion). In addition, significant increases in the uptake of adenosine (fig. 1), hypoxanthine (fig. 2) and inosine (fig. 3) by infected erythrocytes and free parasites as compared to normal erythrocytes were demonstrated. The relative uptake value by free parasites for these substrates at a concentration of 0.01 mM are as follows: Adenosine < Hypoxanthine < Inosine < Adenine.

The uptake of ^3H -labelled 0.01 mM adenosine, hypoxanthine, inosine and adenine by normal erythrocytes, Plasmodium berghei-infected erythrocytes and free parasites were measured in the presence of increasing concentration of unlabelled adenosine, hypoxanthine, inosine and adenine as inhibitors at a maximum inhibitor to substrate ratio of 100:1. The uptake of 0.01 mM ^3H -adenosine by normal erythrocytes was unaffected by unlabelled hypoxanthine or adenine (Fig. 4). The uptake velocity of ^3H -hypoxanthine was significantly inhibited by unlabelled hypoxanthine. However, unlabelled adenosine, inosine and adenine had no effect. Labelled adenine was also maximally inhibited by itself at a 100:1 inhibitor to substrate ratio but was

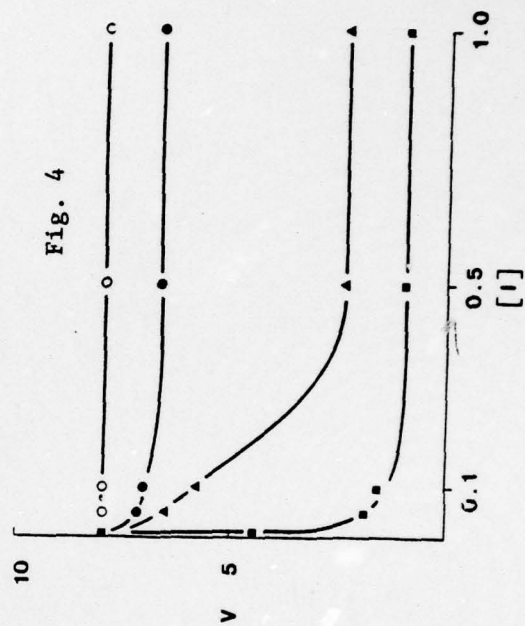
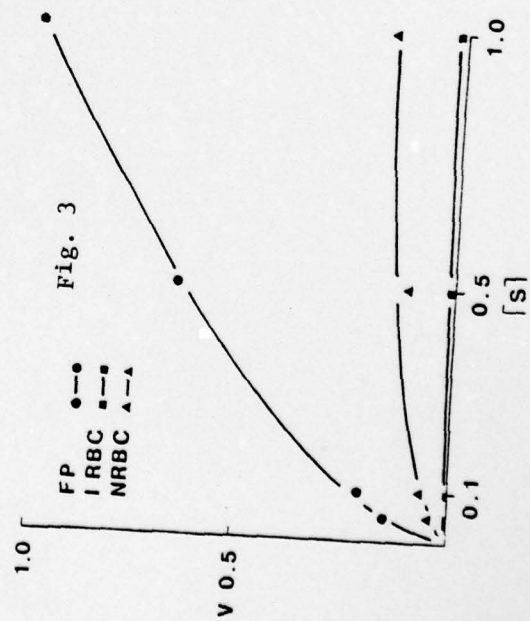
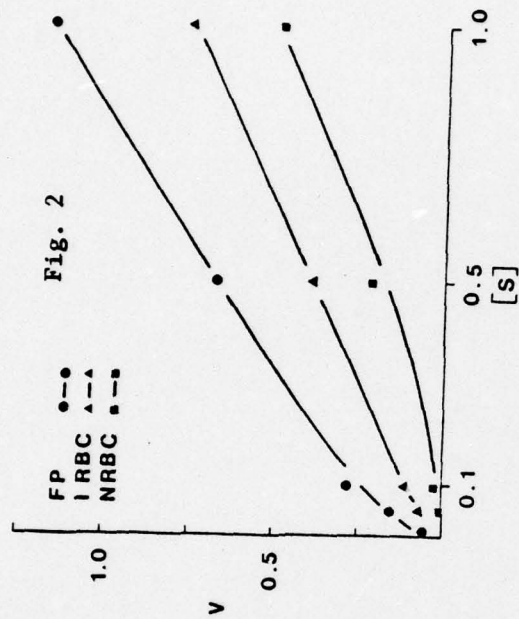
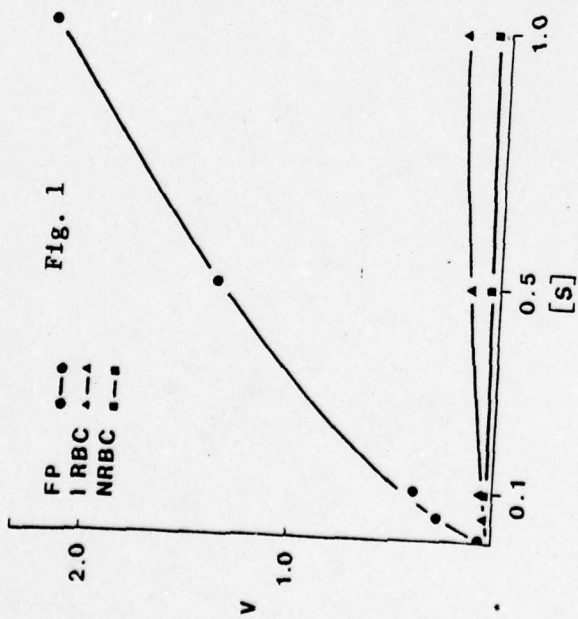


Figure 1. The velocity of ^3H -adenosine uptake (V , $\mu\text{moles/g protein/2 min}$) by normal erythrocytes (\blacksquare), *P. berghei*-infected erythrocytes (\blacktriangle) and "free parasites" (\bullet) as a function of substrate concentration (S , mM). Each point is the mean of three replicates and the lines were fitted by inspection.

Figure 2. The velocity of ^3H -hypoxanthine uptake (V , $\mu\text{moles/g protein/2 min}$) by normal erythrocytes (\blacksquare), *P. berghei*-infected erythrocytes (\blacktriangle) and "free parasites" (\bullet) as a function of substrate concentration (S , mM). Each point is the mean of three replicates and the lines were fitted by inspection.

Figure 3. The velocity of ^{14}C -inosine uptake (V , $\mu\text{moles/g protein/2 min}$) normal erythrocytes (\blacksquare), *P. berghei*-infected erythrocytes (\blacktriangle) and "free parasites" (\bullet) as a function of substrate concentration (S , mM). Each point is the mean of three replicates and the lines were fitted by inspection.

Figure 4. The velocity of $0.01 \text{ mM } ^3\text{H}$ -adenosine uptake (V , $\mu\text{moles/g protein/2 min}$) by normal erythrocytes as a function of increasing concentrations of unlabeled adenine (\circ), hypoxanthine (\bullet), inosine (\blacktriangle) and adenosine (\blacksquare) as inhibitors ($[I]$, mM). Each point is the mean of three replicates and the lines were fitted by inspection.

unaffected by other unlabelled purines. Inosine was inhibited by unlabelled inosine and adenosine but unaffected by hypoxanthine or adenine. These data are summarized in table 1. The results indicate that adenosine may be transported across the plasmalemma of the normal erythrocyte by a common mediated transport system while hypoxanthine and adenine are transported at separate individual loci.

Significant changes in these purine transport interactions were observed upon infection of the erythrocyte with Plasmodium berghei. The uptakes of labelled adenosine, inosine and hypoxanthine were strongly inhibited by all three of these substrates as unlabelled inhibitors. This would suggest a common transport locus for all three substrates. These data are summarized in Table 2. Similar interactions were observed for free parasites and the data summarized in Table 3.

Manandhar and Van Dyke (1975) have demonstrated that adenosine may be deaminated to inosine with subsequent hydrolysis at or on the surface of the plasmodial membrane, hypoxanthine being the pivotal purine for transport. If such a mechanism is indeed operating, one would anticipate our observed decrease in adenosine, inosine and hypoxanthine uptake in the presence of any of these unlabelled substrates as inhibitors. To further substantiate this type of mechanism, the following experiments are currently being conducted in our laboratory.

1. A double isotope technique will be used to follow the fate of purine and ribose moieties of adenosine. ^{14}C -adenosine labelled only in the base and ^3H -adenosine labelled 50% in the base and 50% in ribose will be mixed so that the ratio of DPM/min ^{14}C will be 10:1. If the ratio of the labels remain the following uptake, the data would indicate the intact transport adenosine and probable conversion to AMO. On the other hand, if the ratio decreases following uptake, one might assume deamination and hydrolysis of adenosine to hypoxanthine.

2. A potent adenosine deaminase inhibitor (2-deoxycoformycin) will be added to the malarial cell suspension containing ^3H -adenosine. If the uptake of the tritium label is prevented, the data would suggest the inhibition of adenosine deamination and provide further evidence of the pathway proposed by Manandhar and Van Dyke (1975).

Table 1

The effect of unlabelled substrates on the uptake of ^3H -labelled purines in normal rat erythrocytes. The numbers represent the percent inhibition of the mediated component at a 100:1 inhibitor to substrate ratio with respect to the absence of the inhibitory purine.

^3H -purine	Percent inhibition by unlabelled Substrate (1 mM)			
(0.01 mM)	<u>Adenosine</u>	<u>Hypoxanthine</u>	<u>Inosine</u>	<u>adenine</u>
Adenosine	100	14	93	12
Hypoxanthine	18	100	13	0
Inosine	100	10	100	0
Adenine	0	0	0	100

Table 2

The effect of unlabelled substrates on the uptake of ^3H -labelled purines in the *Plasmodium berghei*-infected erythrocytes. The numbers represent the percent inhibition of the mediated component at a 100:1 inhibitor to substrate ratio with respect to the absence of the inhibitory purine.

^3H -purine	Percent inhibition by unlabelled substrate (1 mM)			
(0.01 mM)	<u>Adenosine</u>	<u>Hypoxanthine</u>	<u>Inosine</u>	<u>Adenine</u>
Adenosine	100	75	100	10
Hypoxanthine	100	100	45	0
Inosine	100	90	100	0
Adenine	18	11	5	100

Table 3

The effect of unlabelled substrates on the uptake of ^3H -labelled purines in free parasites. The numbers represent the percent inhibition of the mediated component at a 100:1 inhibitor to substrate ratio with respect to the absence of the inhibitory purine.

^3H -purine	Percent inhibition by unlabelled substrate (1mM)			
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(0.01 mM)	<u>Adenosine</u>	<u>Hypoxanthine</u>	<u>Inosine</u>	<u>Adenine</u>
Adenosine	100	100	95	
Hypoxanthine	95	100	90	
Inosine	90	95	100	
Adenine	0	0	0	

2. Biochemical effects of selected antimalarials on thyroid function in the rat.

Previous studies showed that administration on the 4-quinolinemethanols, mefloquine (WR 142490) and WR 30090, produced a depression of serum thyroxine levels. Work was continued to evaluate the effects of the 4-quinolinemethanols on thyroid function as a mode of action of this class of drugs. Rats were administered orally 30 mg/kg and 120 mg/kg bodyweight of the antimalarial drug for 5 days. They were sacrificed on day 6 and the blood and tissues collected. Serum T₄, T₃ uptake and TSH were determined by RIA. Body and thyroid weights were recorded.

Results of thyroid function tests after drug administration are shown in Table 4. The serum T₄ was decreased significantly when compared with controls, by both WR 142490 and WR 30090 at doses of either 30 or 120 mg/kg body weight. The serum T₃ was decreased significantly also except for the 30 mg/kg dose of WR 142490. T₃ uptake, a measure of protein combining, was unchanged by these drugs. TSH levels were measured only after 30 mg/kg of WR 142490, but were significantly increased ($p < .001$) at that drug concentration. Thyroid weights per 100 g body weight increased significantly ($p < .05$). Chloroquine and WR 33063, a phenanthrenemethanol, at similar dose regimens did not affect significantly the serum T₄ and T₃ levels.

Studies indicate a direct relationship between P. berghei infection and thyroid function in mice and rat. Shoemaker (1974) reported that pretreatment of mice with propylthiouracil (PTU), a thyroid blocking agent, prior to infection suppressed parasitemia and lengthened survival time. This has been confirmed in our laboratory. The pretreatment of mice prior to P. berghei infection with 5µg/day T₄ for 1 week increased parasitemia and shortened survival time, (Table 5), while the P. berghei infection in rats produced a rapid fall in serum T₄ levels to below detection levels and a progressive decrease in T₃.

Table 4

Serum levels of T₄, T₃, and TSH and T₃ uptake after the administration of 4-quinolinemethanols to rats.

<u>Drug administered in mg/kg body weight</u>					
Test	WR142490			WR30090	
	0	30	120	30	120
T ₄ ug/dl	9.9±3.1	7.3±1.8 p<.01	4.5±1.7 p<.01	4.7±1.4 p<.001	4.1±0.8 p<.001
T ₃ ng/dl	65±16	52±17 n.s.	33±17 p<.001	31±1 p<.001	26±5 p<.001
T ₃ up- take ng/dl	91±7	89±15 n.s.	87±12 n.s.	93±20 n.s.	85±12 n.s.
TSH units	327±33	1520±448 p<.001			

Table 5

Survival Time of Mice Infected with *P. berghei*

Experimental Conditions	Mean Survival Time In Days
Infected Control	6
Treatment with T ₄	4.5
Treatment with PTU	7.2

3. Drug in experimental infections with or Trypanosoma rhodesiense.

Functional impairment of the liver in malaria, as well as trypanosomiasis, has been adequately documented. Nevertheless, relatively little information exists concerning the effects of these diseases states on drug metabolism. Within recent years, it has become increasingly evident that most drugs are metabolized primarily in the liver by enzymes comprising the mixed-function oxidase system. The present study was undertaken to determine in infection with *Trypanosoma rhodesiense* or *Plasmodium berghei* in the rat is accompanied by changes in rats of metabolism of selected drugs.

Studies were initiated to investigate the drug metabolizing ability of the host during infection with *P. berghei*. The ability to metabolize a drug could influence effective drug therapy. Drugs that depend upon

metabolic conversion for activity would have their effectiveness diminished, while these active per se may reach toxic level do to a diminished rate of biotransformation. Preliminary studies were conducted by comparing paralysis time of rats and mice after the I.P. injection of Zoxasolamine (100 mg/kg body weight). Zoxasolamine is a central nervous depressant which is rapidly metabolized (Burns, J.J. et. al 1958). When non-infected rats were compared with rats infected with P. berghei, it was found a prolonged paralysis time for the infected rats. A similar experiment with mice showed a direct correlation between paralysis time and percent of parasitemia, which indicated a progressive decrease in drug metabolism with increasing parasitemia. Also, both Zoxasolamine paralysis time and ethanol toxicity were extended over controls in Trypanosoma rhodesiense in the rat. Work will be continued with emphasis on the role of the microsomal enzymes.

4. Lipid metabolism in malaria: the identification and quantitation of fatty acids.

The source, uptake, and utilization of lipids by the malarial parasite and the effects of the antimalarial drugs on these processes need clarification. Recent technological advance in gas chromatography, which include pyrolytic methylation and increased sensitivity, have made these studies feasible. Studies underway involve the extraction and the chromatography of the fatty acids from the plasma and erythrocytes of non-infected and P. berghei infected rats and from free parasites. Fatty acid of carbon lengths of C₁₄ to C₂₀ and having 1 to 4 unsaturated sites have been identified; other structure will be identified, C₂₀ to C₂₄ with 1 to 6 unsaturated sites, as standards are available. The separation of the octadecanoic fatty acids, oleic and cis vacenic, is being developed since these fatty acid are found in malaria infections. With the use of an internal standard, heptadecanoic acid, the sensitivity of the method is 2 to 5 ug lipid on column.

5. Gluconeogenesis in experimental Trypanosomiasis.

Terminal hypoglycemia accompanied by reduced or depleted hepatic glycogen reserves is a prominent feature of African trypanosomiasis in various experimental animals. Excessive levels of blood lactic acid have also been observed. However, the mechanisms underlying these metabolic changes remain obscure. In this regard, glucose

consumption by the parasites is generally regarded as an insignificant contributory factor.

Blood glucose homeostasis, under the influence of several regulatory hormones, is essentially based on the following metabolic events: 1) release from performed glycogen; 2) recycling of glucose-derived intermediates such as lactate, pyruvate and glycerol; and 3) de novo glucose synthesis from precursor amino acids. Accordingly, the present study was undertaken to examine the possibility that terminal hypoglycemia in experimental trypanosomiasis is basically associated with an impairment of hepatic gluconeogenesis.

Clone-derived stabilates of Trypanosoma rhodesiense (Wellcome strain) were used to produce fulminant and rapidly fatal infections in rats, usually of 3 days duration. Inoculations were administered intraperitoneally and contained approximately 5×10^5 trypanosomes. Plasma glucose values in these animal on the last day of infection were usually one-third to one-fourth the values obtained from non-infected controls. Other determinations, currently in progress, pertain to hepatic glycogen content and the levels of activity of four obligatory gluconeogenic enzymes, namely, glucose 6 - phosphatase, fructose - 1,6 - diphosphatase, phosphoenolpyruvate carboxy kinase, and pyruvate carboxylase. Recently, a more chronic form of the infection produced by the EATRO 1886 strain of T. rhodesiense was also included in this study. This will permit analyses of sequential changes, if any, occurring in various gluconeogenic mechanisms during the course of infection.

6. Phthalate plasticizers in biological fluids.

The confusion regarding phthalate toxicity has cleared somewhat in the wake of rather extensive research during the last five years, and most of the remaining allegations of phthalate toxicity are largely conjectural. While phthalates, in general, and bis-diethyl hexyl phthalate (DEHP), in particular, are regarded as relatively non-toxic, concern has been repeatedly expressed for the large amounts of DEHP found in whole blood and plasma stored in polyvinyl chloride containers. The belief that DEHP may be converted to some more toxic metabolite has prompted studies in rats and ferrets. Although some morphological and histological changes were observed in these animals (Lake, 1975) after daily administration of DEHP for 21 days, no symptoms of acute intoxication were evident at any time. In this regard,

only one group of European researchers have reported possible toxicity with phthalates (Neergaard, 1971). They associated diethyl phthalate with the occurrence of hepatitis in four patients who were undergoing hemodialysis at the time. Diethyl phthalate was found in the polyvinyl chloride tubing used in the procedure, and the hepatitis was attributed to this plasticizer because it was unique to a system and no further problems were experienced once the tubing was replaced. However, diethyl phthalate is not commonly found in polyvinyl chloride tubing used for medical purposes, and it has not been reported as a metabolite or metabolic intermediate. In some instances mono-ethyl hexyl phthalate has been detected in urine from animals after a high dose regimen over an extended period. However, in our work with blood and plasma from humans, only DEHP has been found.

Reported data on the amount of DEHP present in blood stored in polyvinyl chloride bags are consistent. For example, twenty-one days after being stored at 4° C, whole blood was found to contain 0.05-0.07 mg. DEHP per ml., and plasma concentrations were as high as 0.20 mg. per ml. The plasticizer tends to concentrate in the lipoprotein fractions of the blood (Jaeger, 1970; Jacobson, 1974). Expired human whole blood obtained from the blood bank contained 0.064 mg. DEHP per ml which agrees with our data. When this blood was perfused through a pig liver to determine what metabolites might be formed, the level of DEHP dropped to 0.014 mg. per ml. Although the superior vena cava was ligated, the observed drop in DEHP blood level could be attributed, in part, to dilution with blood already in the pig's liver. However, considering the large amount of blood perfused (more than a liter), it is more likely that most of the DEHP was actually removed by the liver. We observed a similar decrease in blood level of DEHP after a unit of blood plasma was administered to a patient during hemodialysis. The plasma, as it came from the polyvinyl chloride container, contained 0.082 mg. DEHP per ml., and this produced an increase in the patient from 0.53 ug/ml to 2.70 ug/ml. Within an hour, the level had dropped about 50% to 1.30 ug, DEHP per ml. No metabolites were detected in the blood from the patient, or from the blood perfused through the pig liver. DEHP has been reported, by other investigators (Jaeger, 1972), to be stored intact in the liver. This conclusion was based on the fact that human tissue obtained at autopsy was found to contain small amounts of the plasticizer although the deceased did not receive any recent transfusion or had any history of hemodialysis. DEHP is readily excreted

intact, and when rats were given ^{14}C labeled DEHP, all of the radioactivity could be accounted for in the urine and feces within the first 96 hours. Very little radioactivity remained in the tissues. While small amounts of the DEHP appears to be retained in lipid deposits of tissues, there is no evidence of any significant accumulations.

Although there seems to be no imminent health threat from the use of polyvinyl chloride products where normal human beings are concerned, the possibility that subtle toxicity may become more pronounced in debilitated persons has prompted further study on patients with renal disease or injury. Work continues in an effort to complete analyses on a series of plasma specimens from kidney transplant patients. If the data from these patients are not significantly different from the data already available on normal patients, the study will be terminated, at least until a specific toxic agent can be determined that relates to DEHP.

7. Study of Drug Metabolites by mass spectrometry: (a) Terbutaline (b) Methaqualone.

(a) The recent introduction of terbutaline, -(3',5'-dihydroxyphenyl)-2-(ter. butylamino) ethanol, and its widespread use in the treatment of asthma has stimulated interest in the pharmacodynamics of this drug. Efforts are being made to develop more simple, routine methodology to pursue these studies, but at the moment, gas chromatography with chemical ionization mass spectrometry seems to be the only viable means to reliably detect the few nanograms of drug in plasma. Moreover, there is indication of a metabolite being present in the plasma, and this is being further investigated in an attempt to correlate this information with the levels of unchanged drug. Preliminary attempts at extraction of the drug from plasma utilizing ion-pair technology were encouraging, and although chemical ionization-mass spectrometry has not been applied as yet in our work, excellent mass spectra were obtained in the electron impact mode with 100 ng. However, the limit of sensitivity was about 20 ng which is not adequate to detect therapeutic doses.

A number of plasma specimens for this study have already been collected, and the analysis of these samples will commence as soon as standardization procedures have been completed on the mass spectrometer in the chemical ionization mode.

(a) Some work still continues on methaqualone,

because specimens from a case of methaqualone intoxication were made available for study. The documentation of the circumstances and sample collections, as well as the fact that the individual survived, present an interesting follow-up to the data published in our previous study. Analyses have already been completed on the specimens submitted, and the data are being prepared for publication.

To summarize these data, blood contains unchanged methaqualone and 2-methyl-3-[2-(hydroxymethyl) phenyl]-4 (³H)-quinazolinone (metabolite I) after an acute intoxication, and the latter is present in higher concentrations than the methaqualone. This relationship persists while the person remains in deep coma, after which the concentration of metabolite I decreases below that of the methaqualone as the patient begins to respond to stimuli. 2-methyl-3-O-tolyl-6-hydroxy-4(³H)-quinazolinone (metabolite V), which is normally excreted in urine after a therapeutic dose, is not excreted to any measurable extent in either the blood or urine. 2-methyl-3-(2-methyl-4-hydroxy phenyl)-4(³H)-quinazolinone (metabolite IV) appears to be the principal metabolite in the urine, and the large amount of this metabolite could be dose related. Finally, our observations that 2-hydroxy methyl-3-O-tolyl-4(³H)-quinazolinone (metabolite II) is present in comparatively high concentrations only a few hours after injection of the drug, were confirmed by data from this case. The metabolite pattern following intoxication appears to be a sharp contrast to that produced by therapeutic doses. This tends to reinforce our contention that an analysis for the metabolites is as important as the analysis for unchanged drug in attempting to establish the severity of an intoxication.

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Publications

1. Sleeman, H.K. and Crossen, H.J. :The Effects of the Antimalarials, 4-Quinolinemethanols, on Thyroid Function in Rats. Abst. National Meeting American Chemical Society, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL	
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23. KEYWORDS (Precede EACH with Security Classification Code) ^a (U) Malaria; (U) Drug Resistance; (U) Chemotherapy; (U) Immunology; (U) Human Volunteer; (U) Monkey; (U) Vectors							
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<p>23. (U) To determine the effect of conventional and experimental antimalarials in the treatment, prophylaxis, and transmission of drug-resistant falciparum malaria. To define vector species and bionomics which influence the transmission of malaria and to develop rationales for vector control. To characterize the cellular immune response of patients infected with malaria. To establish strains of human plasmodia in continuous in-vitro culture. To evaluate candidate antimalarial drugs against simian malaria.</p> <p>24. (U) US Army investigational antimalarial drugs are compared with standard drugs in treatment of drug resistant falciparum malaria in hospitalized human volunteers. Lymphocytes from malaria-infected patients are isolated and their response to malarial antigens characterized. Continuous in-vitro culture of <i>P. falciparum</i> is performed. Chemotherapeutic drugs are studied in rhesus monkeys with <i>P. cynomolgi</i>.</p> <p>25. (U) 77 10 - 78 09 Mefloquine treatment of falciparum malaria continued to result in radical cure of all patients studied. Mefloquine also cured acute attacks of vivax malaria, although it was not sporonticidal against either infection. Fansidar in two dosages was found to be ineffective in terminating the acute attack of vivax malaria. <i>P. falciparum</i> but not <i>P. vivax</i> gametocytemia was stimulated by Fansidar, an effect which may contribute to the spread of falciparum infections. An <i>in vitro</i> microtechnique for the determination of chloroquine resistance in <i>P. falciparum</i> was more useful than the standard technique. Characterization of the response of lymphocytes for malaria-infected patients to non specific mitogen and malaria antigen continued. Isolation of malarial antigen, starting with a technique for production of erythrocyte-free intact parasites, was in progress. The rhesus monkey cynomolgi system was used to test 70 drugs for schizonticidal and/or radical curative effects. Final definitive testing of WR225448 in this system was continuing. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77-30 Sep 78.</p>							

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Project 3M162770A803 MALARIA PROPHYLAXIS

Work Unit 089 Field Studies on drug resistant malaria (AFRIMS)

Investigators.

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1. Sporontocidal Effect of Antimalarials in P. vivax Malaria

OBJECTIVE: To determine the effect of several therapeutic regimens upon the sexual and sporogonic cycles of Plasmodium vivax.

BACKGROUND: In the therapy of malaria, three effects are hoped for. First, the antimalarial should terminate the parasitemia and symptomatology of the acute attack. Secondly, the exoerythrocytic phase of relapsing infections should be eliminated in order to obviate the possibility of relapse. Thirdly, transmission of the infection by a vector mosquito from the patient under therapy to an uninfected individual should be prevented; i.e., gametocytes should be eliminated or rendered non-infectious. The first effect has been studied and reported upon elsewhere in this Annual Report. The second effect has not been examined at this facility. The third effect is the subject of the current report.

Treatment of falciparum malaria with Fansidar, and other sulfonamides, has been seen to result in an increase in gametocyte-positive individuals as well as an increase in the intensity of gametocytemia. Fansidar has been used in great quantities recently in Thailand for the therapy of malaria, both falciparum and vivax. Whether gametocyte stimulation occurs with P. vivax has not previously been investigated.

Mefloquine hydrochloride, a new antimalarial developed by the U.S. Army's Drug Development Program, has not been studied in the field in vivax infections. Its effect upon gametocytemia and infectivity of gametocytes has not been evaluated previously.

METHODS: The study was initiated at the Phrabuddhabat Hospital, Saraburi Province and continues at the Phraya Paholpolpayuhasena Hospital in Kanchanaburi Province. The usual conditions for admission of patients to AFRIMS therapeutic trials were observed. Patients were assigned to therapy groups using one of the following treatment regimens: Mefloquine hydrochloride, Fansidar in either a two-tablet or a three-tablet dose, chloroquine, chloroquine plus a short course of primaquine, and pyrimethamine alone.

Before the initiation of therapy, and on Days 1, 7, 14 and 21 after therapy, 60 laboratory reared Anopheles balabacensis and 60 Anopheles maculatus (IMR strain) were allowed to feed on patients. Two species of vector mosquitoes were used for comparison of susceptibility. Ten mosquitoes from each species were withheld for determination of longevity (subject of a separate report).

TABLE 1

P. vivax Development in Vector Mosquitoes
Before and After Antimalarial Therapy

a. Before Treatment (Day 0)

Therapy	<u>An. balabacensis</u>		<u>An. maculatus</u>	
	Oocysts	Percent Positive	Oocysts	Percent Positive
Mefloquine	33	27	23	23
Fansidar (2 tablets)	13	13	6	6
Fansidar (3 tablets)	26	13	16	17
Chloroquine	29	29	19	15
Chloroquine + Primaquine	26	19	22	13
Pyrimethamine	32	29	37	32

b. After Treatment (Day 1)

Mefloquine	41	13	16	8
Fansidar (2 tablets)	23	20	17	11
Fansidar (3 tablets)	43	56	62	40
Chloroquine	7	5	3	3
Chloroquine + Primaquine	0	0	0	0
Pyrimethamine	39	40	26	31

TABLE 2

Percent of Patients Giving Rise to At Least
One Positive Mosquito Feed

Therapy	Before Therapy (Day 0)	After Therapy (Day 1)
Mefloquine	47%	41%
Fansidar (2 tablets)	25%	50%
Fansidar (3 tablets)	64%	67%
Chloroquine	48%	22%
Chloroquine + Primaquine	56%	4%
Pyrimethamine	60%	40%

Mosquitoes were also fed on uninfected volunteers for simultaneous control. Mosquitoes were dissected 7 and 14 days after feeding. Guts and glands were examined for oocysts and sporozoites, and oocyst indices and sporozoite densities were determined.

RESULTS: The groups were similar in the rate of positivity of mosquito feeds, both in oocysts and sporozoites, prior to initiation of therapy (Table 1). In general, Anopheles balabacensis was more likely to become infected than Anopheles maculatus fed upon the same patient. The group treated with two tablets of Fansidar were less likely to infect mosquitoes fed before treatment, however. There is no obvious reason for this finding.

After therapy, on day 1, patients treated with Fansidar or with pyrimethamine alone were more likely to give rise to mosquito infections than patients treated with mefloquine, chloroquine, or chloroquine plus primaquine. Patients who had been treated with primaquine in addition to chloroquine infected no mosquitoes, even after only a single dose of primaquine (15 mg.). The three tablet dose of Fansidar has an apparent association with increased mosquito positivity.

As expected, gametocytes disappeared quickly after therapy, along with the asexual forms. Except for patients in the two-tablet Fansidar group who were positive on day 7 and patients who relapsed there were no gametocyte-positive patients available for mosquito feeding after day 1.

When patients were examined in terms of producing at least one positive mosquito infection (Table 2) groups were once again similar prior to therapy, with the note again that the group who later received two tablets of Fansidar produced a generally lower infectivity rate. After therapy, fewer patients from the chloroquine and chloroquine- primaquine groups infected mosquitoes. In fact, the single dose of primaquine was associated with only one mosquito infection.

Data are still being collected from this study and a similar examination of P. falciparum is underway.

2. Treatment of the Acute Attack of Malaria Caused by Plasmodium vivax: A Comparison of Mefloquine with Standard Therapy

OBJECTIVE: To compare the effect of several antimalarials upon sexual and asexual parasitemia with P. vivax in naturally infected individuals.

BACKGROUND: Standard therapy for P. vivax infections in Thailand consists of 1,500 mg. of chloroquine administered over the course of three days in G-6-PD normal individuals. Other therapy occasionally used includes Fansidar (sulfadoxine-pyrimethamine), and in some cases pyrimethamine. Mefloquine hydrochloride, a new antimalarial developed by the U.S. Army's Antimalarial Drug Development Program, has been shown to be useful in the therapy of the acute vivax attack in American volunteers (1). It had no effect in the prevention of relapse; however, Mefloquine has also been shown in several studies to be highly effective both in laboratory-induced and naturally acquired infections of P. falciparum.

Primaquine, aside from its activity in the prevention of relapse of infections with P. vivax, is also gametocytocidal against the sexual forms of both P. vivax and P. falciparum. The gametocytocidal effect is apparent after very small doses of the preparation.

METHODS: The study has been underway at two malaria-endemic areas in Thailand. The project was initiated at the Phrabuddhabat Hospital, in Saraburi Province and later moved to the Phraya Paholpolpayuhasena Hospital, The Kanchanaburi Provincial Hospital. Patients were admitted either from the out-patient department of the hospital or from the passive detection center of the National Malaria Eradication Project. The usual conditions for acceptance of subjects to chemotherapeutic trials were applied. Patients were randomly assigned to one of the following therapeutic regimens:

1. Mefloquine hydrochloride, single dose 1,500 mg., p.o.
2. Fansidar, single dose: a) two tablets, b) three tablets.
3. Chloroquine 1,500 mg. Total dose p.o., administered over three days.
4. Chloroquine, 1,500 mg., as above plus primaquine 15 mg. daily for five days.
5. Pyrimethamine, in dosages ranging between 50 and 150 mg.

Patients were normally retained in the hospital until clearance of parasitemia and clinical symptoms. They were followed weekly for 28 days, and at their final visit, they were given primaquine 15 mg. daily for 14 days.

Figure 1

Therapy of the Acute Attack of Vivax Malaria

Therapy	Number	Mean initial asexual parasite count/mm	Mean fever clearance time in hrs.	Mean parasite clearance time in hrs.	Number of treatment failures*
Mefloquine	31	5885	37	48	0
Fansidar (2 tablets)	10	7441	68	77	4
Fansidar (3 tablets)	11	7342	49	93	0
Chloroquine	24	10889	39	53	0
Chloroquine + primaquine	26	7813	42	42	0
Pyrimethamine	6	5233	26	90	4

* Failure to clear parasitemia within 7 days of the initiation of treatment. Fever and parasite clearance times for these patients were not included in the computation of mean values.

RESULTS: One hundred thirteen patients have been studied (Figure 1). It soon became apparent that Fansidar, in either dosage, was ineffectual treatment for vivax malaria. The cure rate for the two-tablet dose was unacceptable and parasite clearance time for the three-tablet dose was markedly longer than that for mefloquine or chloroquine. This is the subject of a subsequent report. Pyrimethamine was studied in order to document the resistance of the parasite to this component of Fansidar. Mefloquine was found to be effective in the elimination of parasites and fever in the 31 patients studied. Fever and parasite clearance times obtained with mefloquine were similar to those associated with chloroquine. Pyrimethamine in several dosages was found to be ineffective, confirming pyrimethamine resistance in the strains studied.

Since no well-documented evidence of chloroquine resistance in P. vivax has appeared, chloroquine remains the drug of choice for the termination of the acute attack of vivax malaria. Primaquine must be used subsequently in order to prevent relapse.

When mefloquine is available, it may be useful in the treatment of acute vivax malaria, although its structural similarity to primaquine may make combination therapy with these two agents hazardous, since toxicity may be expected to be additive.

The prolonged half-life of mefloquine in man is currently being evaluated using specimens collected in Bangkok. Patients with P. falciparum infections were treated with mefloquine and then subjected to repeated venipuncture in order to determine the pharmacokinetics of this preparation (Cf. AFRIMS Annual Report 1976-1977). Mefloquine blood levels are currently being estimated on these specimens at the Walter Reed Army Institute of Research.

3. Treatment of Vivax Malaria with Sulfadoxine-Pyrimethamine and with Pyrimethamine Alone

OBJECTIVE: To evaluate the activity of pyrimethamine alone and in combination with sulfadoxine against acute malaria due to P. vivax.

BACKGROUND: Sulfadoxine-pyrimethamine (Fansidar, Roche Laboratories) is used in increasing quantities for the therapy of malaria in areas where P. falciparum is resistant to chloroquine. The combination remains highly effective against asexual forms of P. falciparum. Field studies of hospitalized patients carried out by this Laboratory over the past five years have shown virtually no change in the efficacy of sulfadoxine-pyrimethamine. Radical cure rates

for P. falciparum have ranged between 80 and 91%. Two tablets and three tablets administered as a single dose appear to have equal activity in the adult Thai populations studied (a tablet includes sulfadoxine 500 mg., and pyrimethamine 25 mg.). In view of its efficacy and lack of toxicity, the value of this preparation as a single-dose regimen for mild to moderately severe falciparum malaria is undisputed.

Fansidar has not generally been recommended for the therapy of P. vivax infections although the package insert lists this parasite as one of the indications for use of the combination, along with P. falciparum and P. malariae. However, in areas endemic for chloroquine-resistant falciparum malaria, sulfadoxine-pyrimethamine is widely used for the treatment of fevers presumed to be malaria when microscopic diagnosis is either not available or not convenient. Malaria eradication programs often use sulfadoxine-pyrimethamine as presumptive therapy for fevers assumed to be malaria in areas of known P. falciparum drug resistance, where follow-up is difficult or impossible. In addition, clinics without adequate laboratory support often treat all cases of suspected malaria with sulfadoxine-pyrimethamine. In Thailand, the combination can be purchased by the patient at drug stores and self-treatment may be extremely common. The fact that more than sixteen million tablets of Fansidar were imported into Thailand over a recent one-year period reflects the wide use of the drug.

Vivax malaria has increased in incidence dramatically over the last several years in Thailand. In the Bhu Phram Valley, the proportion of new vivax infections increased from 44% of the population infected in 1972 to 80% in 1976, whereas falciparum infection rates remained nearly unchanged (2, 3). In an attempt to delineate factors which may have contributed to this increase, our laboratory has considered the possibility that treatment with Fansidar may be implicated. Since sulfanamides appear to stimulate the production of infectious gametocytes in P. falciparum the effect of sulfadoxine-pyrimethamine on vivax gametocytemia is being evaluated. During the course of this investigation it became apparent that the combination is not effective therapy for the acute episode of vivax malaria.

METHODS AND RESULTS: In Phrabuddhabat Hospital, Saraburi Province, Central Thailand, ten patients with peripheral smears positive for P. vivax were treated with two tablets of Fansidar (sulfadoxine 1.0 gm., pyrimethamine 50 mg.). Patients were all males, ranging in age from 19 to 33 years (mean 25 years)(Table 3).

Of the ten patients, only six cleared their asexual parasitemia by day 7 following therapy, and mean fever and parasite clearance times were prolonged at 63 and 73 hours respectively. The remaining four patients, although they had a reduction in the level of parasitemia and clearance of fever, still had asexual parasites on their peripheral blood films seven days after treatment. Active sulfonamide was detected in the serum of all patients, indicating adequate drug absorption.

Eleven patients, ranging in age from 19 to 46 years, with a mean of 27 years, were treated with three tablets of the combination, (pyrimethamine 75 mg., sulfadoxine 1.5 gm.). All patients in this group cleared parasitemia within seven days; however, mean parasite and fever clearance times were prolonged at 90 and 50 hours respectively (mean values for 18 patients treated with chloroquine were 52 and 40 hours respectively).

In addition, six patients aged from 19 to 38 years (mean 25 years) were treated with pyrimethamine alone in dosages ranging from a single 50 mg. dose to 50 mg. daily for three days. Of this group, only two had cleared parasitemia by day 7, one patient treated with 50 mg. dose and one patient treated with 50 mg., daily for three days. Parasite clearance times for these two patients were long, 113 and 66 hours. Fever, however, was not prolonged - 32 and 20 hours.

Patients whose parasitemia failed to clear or relapsed were treated with chloroquine 1.5 gm. over three days, followed in G-6-PD normal individuals, by primaquine 15 mg. daily for 14 days. All patients so treated were cured. At the end of the 28 day follow-up period, all G-6-PD normal patients were given a 14-day course of primaquine.

In most cases gametocytes infectious to mosquitoes were present in the peripheral blood as long as the asexual parasites. Therefore, patients treated with sulfadoxine-pyrimethamine are carriers of gametocytes available to infect vector mosquitoes for a longer period of time than patients treated with more effective therapy, e.g., chloroquine. Whether this fact has a significant effect upon the transmission of the parasite is not clear.

Pyrimethamine, and pyrimethamine combined with sulfadoxine, appear to be inappropriate therapeutic regimens for vivax malaria in Thailand. Pyrimethamine alone and the two-tablet dose of

TABLE 3
Results of Treatment of *P. vivax* with Pyrimethamine 50 mg. and
Sulfadoxine 1 gm.

Patient No.	Initial Asexual Count	Fever Clearance Time (hours)	Asexual Parasite Clearance Time (hours)	Serum Sulfonamide Level			Comment
				24 hours after Drug Administration	7-Day Follow-Up		
11-0348	34,390	42	53	13 mg/100 ml	3 mg/100 ml**		
11-0353	6,300	80	No clearance by Day 7*	10 "	6 "		
11-0355	4,452	30	64	-	6 "	**	
11-0366	13,800	80	No clearance by Day 7*	11 "	6 "		
11-0367	8,310	No fever	52	12 "	9 "		
11-0376	3,080	72	No clearance by Day 7*	10 "	6 "		
11-0388	5,796	120	102	12 "	8 "		Relapse Day 21*
11-0399	1,160	28	20	16 "	11 "		
11-0400	1,700	96	140	12 "	8 "		Relapse Day 28*
11-0401	5,360	72	No clearance by Day 7*	11 "	6 "		

*Patients were retreated with Chloroquine-Primaquine
**Day 14

sulfadoxine-pyrimethamine have unacceptable activity in terminating the acute attack. The three-tablet dose of the combination provides clearance of fever and parasitemia, but acts very slowly and cannot be recommended.

Children with P. vivax studied in Bangkok have shown even higher failure rates after treatment with sulfadoxine-pyrimethamine (4). Our higher success rate may reflect the contribution of immunity in adult subjects.

Since there have been no verified reports of vivax resistance to chloroquine, it should be emphasized that whenever species diagnosis of malaria is possible, chloroquine is the drug of choice for termination of the acute vivax attack, particularly in areas where pyrimethamine resistance is suspected or documented. Primaquine, of course, is necessary for the prevention of relapse. Dosage of primaquine depends upon the local prevalence of G-6-PD deficiency and must be adjusted accordingly.

4. In Vitro Evaluation of Antimalarial Drugs Against P. falciparum: A New Technique

OBJECTIVE: To monitor chloroquine sensitivity of strains of P. falciparum. To evaluate the newly described "Micro" technique of Rieckmann.

BACKGROUND: For the past several years, the Department of Medicine has monitored the in vitro susceptibility of P. falciparum to chloroquine in areas where in vivo drug testing has been carried out. The technique used has been that of Rieckmann, et al., (5). Recently, a new technique (6) has been described which is basically similar to the earlier technique but which has been adapted to use "micro" amounts of blood, enabling the test to be performed on finger-prick specimens rather than on larger volumes obtained by venipuncture. The technique of continuous culture of P. falciparum described by Trager, et al., (7) has been applied for the purpose of short term cultivation in assessment of the action of schizontocidal drugs.

METHODS: The two techniques were carried out simultaneously on blood obtained from naturally infected patients presenting to the passive detection center of the National Malaria Eradication Project in Kanchanaburi. The "macro" technique was performed as described in previous publications and Annual Reports. The "micro" technique is performed as follows: chloroquine diphosphate in quantities, ranging from 0.1 to 25 nanograms, is dissolved in 25 microliters of distilled water and added to flat bottomed wells

FIGURE 2. IN VITRO INHIBITION OF SCHIZOGONY BY CHLOROQUINE
"MICRO" Technique

Case No.	Asexual Parasites per mm ³	Control (Schizonts per 200 parasites)	Inhibition of Schizont Development (p-mols chloroquine/well)									
			1	2	4	5	6	8	12	16	32	
22	30016	118	20.3	84.7	74.7	69.4	82.2	72.0	72.8	56.7	21.1	
23	7040	194	90.4	99.2	94.6	95.1	97.1	96.6	99.2	97.1	43.7	
24	4350	127	66.6	84.7	75.2	40.0	90.9	78.4	43.9	19.6	5.4	
25	3480	120	76.6	95.0	74.1	73.3	70.8	81.6	41.6	27.5	0	
29	14950	196	98.4	0	64.7	96.9	77.5	82.1	75.5	67.8	21.4	
45	8200	130	92.3	97.5	110.7	92.3	110.7	90.7	106.1	88.4	22.3	
47	22446	73	100.0	90.4	84.0	34.2	39.7	57.5	38.3	26.0	0	
50	3567	160	88.4	95.3	71.0	39.1	87.8	83.4	70.4	46.1	56.6	
52	3219	168	87.5	88.6	89.8	75.0	90.4	88.6	82.1	37.5	89.7	
54	7287	177	93.2	92.6	84.1	68.3	86.4	79.0	63.8	59.8	3.3	
60	10440	29	98.3	108.4	84.7	64.4	84.7	67.7	47.4	37.2	37.2	
61	2610	160	114.3	106.2	83.1	83.7	91.2	87.5	71.2	70.6	0	
Mean	9800	138	85	87	83	69	84	81	68	53	25	

Case No.	Asexual Parasites per mm ³	Control (Schizonts per 200 parasites)	"MACRO" Technique Inhibition of Schizont Development (n-mols chloroquine/vial)							
			0.5	1	1.25	1.5	2	2.5	3	4
22	30016	61	92.6	73.1	100.8	55.2	47.1	37.3	29.2	4.8
23	7040	189	90.4	75.1	41.2	36.5	28.0	7.9	6.3	2.6
24	4350	166	92.4	43.8	18.0	4.2	1.8	0	0	0
25	3480	150	88.3	53.8	27.2	1.9	1.3	0.6	0	0
29	14950	152	81.5	53.9	37.5	19.0	25.6	0.6	0.6	0
45	8200	123	83.4	42.1	33.1	15.3	4.8	0.8	0	0
47	22446	28	42.8	10.7	3.5	10.7	3.5	0	0	0
50	3567	47	98.9	67.3	58.9	31.5	25.2	29.4	4.2	77.8
52	3219	108	63.8	38.8	32.4	12.0	0	0	0	0
54	7287	120	89.6	92.1	68.8	73.8	51.4	4.1	9.1	0
60	10440	11	36.3	54.5	27.2	54.5	18.1	0	0	0
61	2610	163	72.7	55.0	40.9	38.5	17.7	12.8	24.4	0
Mean	9800	110	78	55	41	30	19	8	6	7

Fig 3. Comparison of Techniques
(Percent Maturation)
Sample/Control

Technique	Number	Mean Asexual Parasite Count/mm ³	Mean Number of Schizonts per 200 parasites	Chloroquine Concentration (nanomols/ml)											
				0.2	0.4	0.5	0.8	1	1.2	1.25	1.5	1.6	2	2.4	2.5
"Micro"	12	9800	138	85	87		83	69	84		81			68	
"Macro"	12	9800	110			78		55		41	30		19		8
															6
															7
															25
															53
															3
															4
															6.5

of micro titer plates. The plates are dried in an incubator and stored. At the time of use, the dried chloroquine is redissolved in 50 microliters of culture medium consisting of RPMI 1640 powdered medium, sodium bicarbonate, HEPES buffer and gentamycin sulphate. Five microliters of parasitized blood is placed in each well using an Eppendorf pipette. The plate is covered with a lid, agitated, and placed in a glass dessicator containing a paraffin candle. After the candle is lit, the dessicator lid is replaced and the fumes are allowed to escape through an open stopcock. The stopcock is closed just before the flame goes out. The dessicator is then placed in an incubator at 38°-39°C for 24-30 hours. After incubation thick blood films prepared from each well are dried thoroughly, stained for 20 minutes with giemsa, and examined for parasite maturation. The number of schizonts per 200 asexual parasites is determined in control and drug samples. Values for samples containing drug are expressed as percentage of control samples.

RESULTS: Results of incubation using both techniques are shown in Figure 1. It is apparent that although cultivation and schizont production were successful using the "micro" technique, inhibition of growth was not noted until much higher concentrations of chloroquine were used. Figure 3 shows a more direct comparison of the two techniques. In this representation of results, amounts of chloroquine have been expressed as nanomoles per milliliter, adjusting for the differing volumes of blood used in the two techniques. It is obvious that in the micro culture system, schizonts continued to be formed at higher concentrations of chloroquine than in the "macro" technique.

Successful short-term cultivation of *P. falciparum* using "micro" amounts of blood is significant for field evaluation of anti-malarials susceptibility. The technique requires further work in our hands before it is as useful as the more established "macro" technique. The project will continue and will be expanded for the evaluation of other drugs.

5. Evaluation of Experimental Antimalarial Drugs in Rhesus Monkeys Infected with Plasmodium cynomolgi (Blood Schizonticidal Tests)

OBJECTIVE: To evaluate the blood schizonticidal activity of selected experimental drugs against *P. cynomolgi* in rhesus monkeys (*Macaca mulatta*).

Table 4. Summary of Blood Schizonticidal Tests in Rhesus Monkeys.

Type of Compound	WRAIR Drug Number	Minimum Curative Dose (mg/kg/day)
4-Aminoquinoline	228258	*TNC
	228979	0.316
8-Aminoquinoline	232584	3.16
	232956	TNC
	233078	3.16
	233195	TNC
	234578	1.0
Quinoline methanols	215440	3.16
Miscellaneous	229049	**NC(100.0)
	231135	10.0

* - TNC - Testing Not Completed- Testing on these drugs had not been completed when the program was suspended.

** - NC - Not Curative - The compound had suppressive activity but did not cure at the maximum dose tested. Maximum dose tested is indicated in parentheses.

BACKGROUND: This is a continuation of studies initiated in 1971. A chronological report of methodology and results are available in SEATO/AFRIMS Medical Research Laboratory Annual Reports, 1971 through 1977. These studies are conducted in association with the Division of Experimental Therapeutics, Walter Reed Army Institute of Research.

METHODS: Experimental drugs were evaluated in rhesus monkeys utilizing various dosage levels. Rhesus monkeys, negative for malaria parasites on prestudy examination, were infected by intravenous inoculation of 5×10^8 parasitized erythrocytes obtained from donor monkeys infected with P. cynomolgi Strain B.

Beginning on post-inoculation day 4, test drugs were administered orally, via gastric intubation, for seven days. Malaria parasitemia was monitored in each monkey by daily bleedings for the first fifteen days following inoculation and then every other day for the remainder of the study. Post-treatment day 20, monkeys positive for malaria parasites were terminated and those negative for malaria parasites were splenectomized. Splenectomized monkeys continuously negative for malaria parasites through post-treatment day 50 were considered cured.

RESULTS: Suppression of parasitemia was indicative of blood schizonticidal activity. Ten experimental drugs were evaluated, with results summarized in Table 4.

Each infected donor monkey was utilized as the vehicle control for the previous drug study. This passage system for the malaria parasite has resulted in 125 monkey to monkey passages of the original P. cynomolgi Strain B parasite used in these studies.

Due to the ban of the exportation of rhesus monkeys by the Indian government, the blood schizonticidal testing was suspended in March 1978. Testing will be continued when additional monkeys become available.

6. Evaluation of Experimental Antimalarial Drugs For Radical Curative Activity in the Rhesus Monkey

OBJECTIVE: To evaluate the radical curative effectiveness of selected experimental drugs in rhesus monkeys (Macaca mulatta) infected with Plasmodium cynomolgi malaria.

BACKGROUND: This is a continuation of studies initiated by this Laboratory in 1974. A chronological report of the methodology and results are available in the SEATO/AFRIMS Medical Research Laboratory Annual Reports, 1975-76 and 1976-77. These studies are conducted in association with the Department of Experimental Therapeutics, Walter Reed Army Institute of Research.

METHODS: Rhesus monkeys were inoculated intravenously with sporozoites produced in Anopheles balabacensis mosquitoes.

An. balabacensis mosquitoes were fed on P. cynomolgi infected monkeys. This feeding was conducted during the second or third rise in parasitemia and when both male and female gametocytes were present as evidenced by a blood smear. On post-feeding day 14, the sporozoites were harvested from the salivary glands of the infected mosquitoes and diluted in a saline-normal monkey serum solution (1:1) to a concentration of $5 - 20 \times 10^5$ sporozoites per ml. Preselected, malaria-negative rhesus monkeys were immediately inoculated with one ml. of the sporozoite solution.

Each monkey was monitored by blood smears daily, beginning on day 7 post-treatment, for the development of a parasitemia. When the parasitemia reached $5 - 25 \times 10^5$ parasites per cmm, drugs were administered daily for seven days at a predetermined dosage level, based on mg of drug/kg of body weight. To permit evaluation of drug activity against tissue parasitic forms independently of blood schizonticidal activity, chloroquine phosphate was administered simultaneously with each test drug at 5 mg/kg body weight/day.

Following administration of the test drug, malaria parasitemia was monitored by examination of giemsa stained blood smears daily for twelve days and on Monday, Wednesday and Friday thereafter. Prior to 1 March 78, monkeys which converted to a negative parasitemia and remained so through post-treatment day 20 were splenectomized and monitored an additional 33 days. Those that remained free of malaria parasites during this period were considered cured. After 1 March 78, monkeys which converted to a negative parasitemia were monitored for 80 days post-treatment with no splenectomy. Those remaining negative during this period were considered cured.

RESULTS: A total of 79 experimental drugs were evaluated; results are summarized in Table 5.

Splenectomy of negative monkeys was discontinued in an effort to preserve the monkeys for further research. This was deemed necessary, especially in light of the ban on the exportation of rhesus monkeys from India. Also due to the ban, a reduction

Table 5. Summary of Sporozoite Induced Tests in Rhesus Monkeys

Type of Compound	WRAIR Drug Number	Minimum Curative Dose* (mg/kg/day)
8 - Aminoquinoline	199981	**NC (10)
	211814	1.0
	214198	NC (10)
	215730	10.0
	219373	10.0
	222890	10.0
	225374	1.0
	225448	0.316
	225845	1.0
	226261	NC (1.0)
	226899	0.316
	226984	1.0
	228000	1.0
	228002	1.0
	228335	1.0
	228583	0.316
	229406	10.0
	230395	10.0
	231030	1.0
	231033	1.0
	232584	0.316
	232956	0.316
	233078	1.0
	233195	1.0
	233537	1.0
	233539	1.0
	233627	1.0
	233821	1.0
	233878	3.16
	233881	3.16
	234099	10.0
	234578	1.0
	234738	3.16
	235202	1.0
	235485	1.0
	235720	10.0
	235724	10.0
	236066	NC (10)
	236645	10.0
	236646	NC (10)

* Administered orally with 5.0 mg/kg/day of chloroquine phosphate.

** Not Curative - The compound did not cure at the maximum dose tested or tolerated. The maximum dose is indicated in parentheses.

Type of Compound	WRAIR Drug Number	Minimum Curative Dose* (mg/kg/day)
Acridines	226970	NC (10)
	227282	NC (10)
	231135	NC (10)
	233599	NC (10)
	233600	NC (10)
	233602	NC (10)
	233626	NC (10)
	233744	NC (10)
	234064	NC (10)
	235471	NC (10)
	235474	NC (10)
	235477	NC (10)
Pteridines	40070	NC (10)
	236062	NC (10)
	236087	NC (10)
2-4 Diaminoquinazoline	150015	NC (10)
	150017	NC (10)
	155004	NC (10)
Naphthoquinone	25175	NC (10)
	49808	NC (10)
Quinolines	212293	NC (10)
	230688	NC (10)
Miscellaneous	6012	NC (10)
	77250	NC (10)
	96345	NC (10)
	190729	NC (10)
	194905	NC (10)
	194965	NC (10)
	203659	NC (10)
	210809	10.0
	228258	NC (10)
	229184	NC (10)
	232439	10.0
	233538	NC (10)
	234852	10.0
	235201	NC (10)

* Administered orally with 5.0 mg/kg/day of chloroquine phosphate.

** Not Curative - The compound did not cure at the maximum dose tested or tolerated. The maximum dose is indicated in parentheses.

in the number of drugs being tested was necessary. Plans are now being formulated to investigate the possibility of other non-human primate hosts, especially the Malaysian Macaca fascicularis (crab eating Macaque).

Problems encountered: The An.balabacensis colony was decimated apparently due to a change in diet. The mosquitoes were fed on rat and mouse chow and during the year the source of this feed was changed from the United States to a local producer. This problem was rectified by returning to the U.S. manufactured rat and mouse chow. During the mosquito to monkey sporozoite passage, SP-30, the malaria sporozoites were lost. After several unsuccessful attempts the sporozoites were recovered by the following procedure:

1. An old sporozoite donor monkey with a low chronic parasitemia was splenectomized and when its parasitemia reached 10^5 parasites per cmm, blood was drawn and inoculated intravenously into two malaria free monkeys.

2. These monkeys were then monitored daily and during the second parasitemia rise with male and female gametocytes present An.balabacensis mosquitoes were allowed to feed.

3. The mosquitoes were handled and the sporozoites harvested and inoculated into two malaria free monkeys in the routine manner.

4. Malaria parasitemia in these monkeys was evidence that the monkey - mosquito - monkey sporozoites had been recovered.

7. Comparative Study of WR 225448 and Primaquine in the Plasmodium cynomolgi - Rhesus Monkey Radical Curative Model

OBJECTIVE:

1. To compare the efficacy of WR 225448-chloroquine with the radical curative combination of primaquine-chloroquine.

2. To compare the efficacy of WR 225448 alone with the combination WR 225448-chloroquine.

3. To determine whether WR 225448 is efficacious in a single oral dose.

BACKGROUND: In rhesus monkeys infected with sporozoites of P. cynomolgi, primaquine (in combination with chloroquine) is a radical curative drug. In this combination, primaquine cures most animals at a total dose of 3.5 mg base per kg body weight, whether that dose is a single dose or divided into 3 or 7 daily doses (8).

In man, the toxicity of primaquine precludes administration in a single curative dose. Thus, to achieve a radical cure of P. vivax in man, the dose is ordinarily given in divided doses over 14 to 21 days (in conjunction with a 3-day course of chloroquine).

WR 225448, an experimental 8-aminoquinoline, appeared to be as potent as primaquine as a tissue schizonticide and had exceptionally potent blood schizonticidal activity. The toxicity of WR 225448 was not investigated, but preliminary evidence suggested that it was at least no more toxic than primaquine.

Because of the apparent blood schizonticidal activity of WR 225448, the ability to achieve a radical cure of sporozoite induced P. cynomolgi without simultaneous administration of a second drug such as chloroquine was considered a real possibility. Since preliminary testing of WR 225448 indicated that it had a better therapeutic index than primaquine, it was decided to also test its efficacy by a single oral dose.

The dose ranges (Table 6) selected for this study were based on assumptions that the new lot of WR 225448 used in this study would have approximately the potency as the succinate salt used in preliminary studies and that WR 225448 would cure at the same total dose whether it was given in a single dose or in seven divided doses.

METHODS: Rhesus monkeys were inoculated intravenously with $5-20 \times 10^5$ P. cynomolgi sporozoites produced in Anopheles balabacensis mosquitoes. Each monkey was then assigned, by random selection, to a particular drug-dose regimen.

Administration of drug in each monkey was initiated on the day after its initial parasitemia reached 5000/cmm (chloroquine was given beginning on this day in the appropriate groups). Parasitemia was determined in each monkey, by blood smears, three times prior to inoculation, daily from day six post-inoculation until three days after parasitemia is suppressed to zero, then every other day through day 40 and twice weekly thereafter. During relapses or recrudescences of parasitemia, counts were made daily.

Monkeys in which parasitemia were cleared by drug were monitored thru day 40 at which time they were splenectomized and then monitored for an additional 40 days. If negative at the end of this period they were considered cured. Monkeys in which parasitemia was not cleared by drug were terminated on day 40.

Monkeys in which the parasitemia was cleared by drug but then reappeared before day 40 were treated with chloroquine phosphate, orally, 5 mg/kg for seven days (whether or not chloroquine was included in the original regimen). If the parasitemia was cleared by the chloroquine, splenectomy was performed 20 days after clearance and the monkey monitored an additional 30 days. Splenectomized monkeys in which parasitemia reappeared were treated with chloroquine phosphate, orally, 5 mg/kg for 7 days. Those animals in which the parasitemia was cleared by this chloroquine treatment were monitored for an additional 50 days.

RESULTS: Results for each treatment regimen are summarized in Table 7. Results of each drug regimen were categorized as Cure, Relapse, or Recrudescence based on the following definitions:

1. Cure: No reoccurrence of parasitemia following original treatment.
2. Relapse: Failure of the curative drug to clear all tissue parasites. Confirmed by administration of chloroquine to all monkeys with recurrent parasitemia followed by a temporary clearance of the parasitemia.
3. Recrudescence: Failure of the curative drug to clear all blood parasites. Confirmed by administration of chloroquine to all monkeys with recurrent parasitemia followed by a permanent clearance of parasitemia.

A combination of WR 225448 and chloroquine was clearly the most potent regimen. In combination with chloroquine, the minimum curative dose of WR 225448 was 0.876 mg base per kg body weight. Administered singly, WR 225448 was not curative at the highest dose utilized, 1.75 mg base per kg body weight. Results indicate that WR 225448 is equally effective as a tissue schizonticide when administered either single or in combination with chloroquine.

Primaquine was not consistently curative at any regimen tested. The lowest dose of primaquine which produced a cure was 3.5 mg/kg body weight. Therefore, using base weight of the drug as the criteria for evaluation, WR 225448 is 4-8 times as potent as

Table 6 Treatment Regimens

Dosage of WR 225448 or Primaquine mg Base/Kg body weight	TREATMENT GROUPS					Dosage of Control mg Base/Kg Body weight		
	WR225448*	WR225448* +Chloroquine**	Primaquine* +Chloroquine**	Primaquine* Chloroquine**	Vehicle**	7.0	3.1	0
7.0			2					
3.5			2					
1.75	2***	2	2					
0.875	2	2						
0.4375	2	2						
				2				
							2	
								2

1. In all cases when Chloroquine was given in conjunction with another drug (WR 225448 or Primaquine) the dose of Chloroquine was 3.1 mg Base/Kg Body weight.

2. * Single oral dose
 ** Seven daily oral doses
 *** Number of Monkeys/dose

Table 7 Summary of Results

TREATMENT GROUPS				CONTROLS		Dosage of Controls mg Base/Kg Body weight	
WR225448*	WR225448* +Chloro- quine**	Primaquine* +Chloroquine**	Primaquine*	Chloroquine**	Vehicle**	7.0	3.1
7.0		G286-Recrudescence*** G308-Relapse					
3.5		G278-Cure G294-Relapse					
1.75	G335-Recrudescence G277-Recrudescence	G267-Cure G307-Cure	G228-Relapse G309-Relapse				
0.875	G273-Recrudescence G261-Recrudescence	G260-Cure G283-Cure					
0.4375	G279-Recrudescence G293-Recrudescence	G272-Relapse G274-Relapse					
			G264-Recrudescence G296-Recrudescence				
				G246-Relapse G262-Relapse			
					G291-No Effect G253-No Effect		0

* - Single oral dose
** - Seven daily oral doses
*** - Monkey number/result

primaquine as a tissue schizonticide when administered as a single oral dose. Both primaquine and WR 225448 were weighed and administered in a similar random manner. One can only speculate in regard to why primaquine in combination with chloroquine was not consistently curative at 3.5 and 7.0 mg base per kg body weight.

Although WR 225448 is more potent as a tissue schizonticide, it does possess blood schizonticidal activity. Previous studies have indicated that WR 225448 is an effective blood schizonticide at 5.5 mg base per kg body weight, but not at 1.75 mg base per kg body weight. Results of this study are corroborative. The blood schizonticidal MCD₅₀ apparently lies between 5.5 and 1.75 mg per kg. A second study using other dose regimens is now being formulated.

8. Lymphocytotoxic Factors in Malaria Patients

OBJECTIVE: To identify lymphocytotoxic activity in the sera of patients infected with falciparum or vivax malaria.

BACKGROUND: There have been several reports in the literature of serum associated antibodies which are cytotoxic for autologous lymphocytes. Terasaki et al. reported such activity in sera from patients afflicted with either systemic lupus erythematosus or rheumatoid arthritis (9). These findings were confirmed by Michlmayr et al. (10) who indicated that both T lymphocytes and B lymphocytes were target cells although primarily the former were killed. Dettoratus et al. (11) extended these findings with their report of lymphocytotoxicity in patients infected with hepatitis. Earlier work in this laboratory (12) indicated a marked suppression in the proportion of circulating T lymphocytes during infection with malaria. It was reasoned that lymphocytotoxicity might be one mechanism underlying this phenomenon. Following earlier attempts (Annual Report 1977) which had proved fruitless, we again assayed for cytotoxic activity with modified technique as reported herein.

METHODS: Cytotoxic assays were performed with the sera of patients infected with either falciparum or vivax malaria. The methodology of Terasaki (9) was utilized with modification. Control sera and target lymphocytes were obtained from normal volunteers. Mononuclear leukocytes isolated by ficoll hypaque centrifugation were adjusted to a concentration of 3×10^6 cells/ml in HBSS (pH 7.3) with 10% fetal calf serum. Experiments were conducted in flat bottom tissue culture trays. Each experimental set-up included 0.1 ml each of cell suspension, human serum and undiluted rabbit

Table 8. Lymphocytotoxins in Malaria

	<u>P. falciparum</u> (57)		<u>P. vivax</u> (50)		<u>Controls</u> (29)	
	<u>15°C</u>	<u>37°C</u>	<u>15°C</u>	<u>37°C</u>	<u>15°C</u>	<u>37°C</u>
Range:	3.5-72*	2.5-24	4.5-78	3-32	2.5-4.5	2.5-4.5
Mean:	19.6	6.9	29.5	8.5	3.3	3.4
SD:	14.8	4.6	10.7	6.4	0.6	0.5

*Percent Cytotoxicity by Dye Exclusion

serum (as a source of complement). Assays run at 15°C were done in a refrigerated centrifuge while those at 37°C were done in an incubator. Total incubation times were 4 hours. The percent of dead cells per 200 mononuclear cells were determined in duplicate by eosin dye exclusion. All counts were made "blind" with the identity of the sera unknown to the investigator.

RESULTS: Serum associated cell death was demonstrated both at 15°C and 37°C - particularly at the lower temperature. Table 8 summarizes the results of these experiments. In fifty-seven sera from patients infected with *P. falciparum* there was an average percent cell death of 19.6 at 15°C and 6.9 at 37°C compared with 3.3 and 3.4 percent respectively for the normal control sera. The range of killing in the patient sera was considerable. There was no apparent relationship between the level of infection and cytotoxicity. Values for the *P. vivax* sera were higher with a mean of 29.5 percent cell death at 15°C and 8.5 percent at 37°C. It is possible that this phenomena may, in part, serve to modulate cellular response to human malaria. This is a final report pending the re-design of protocols for more definitive investigations.

9. Responsiveness of Malaria Patient Leukocytes in Mixed Leukocyte Culture (MLC)

OBJECTIVE: To evaluate general responsiveness of malaria patient leukocytes to stimulation with allogeneic white cells.

BACKGROUND: The responsiveness of one population of white cells to culture with those of an allogeneic source is well established. This methodology has been applied primarily in tissue typing research utilizing in vitro cellular recognition (stimulation) as a parameter for predicting in vitro organ transplant rejection. More recently, the characteristics of cells participating in this reaction (13, 14) and factors modulating the reaction (15) have been described.

METHODS: Mononuclear cells were isolated by ficoll hypaque centrifugation according to the methodology of Boyum (16). Stimulator cells were prepared by treating cells in RPMI 1640 media with 50 ug/ml mitomycin C at 37°C for 45 minutes. After washing (x3) in SBSS the cells were adjusted to 2.5×10^6 /ml in media. In the initial experiments one way cell crosses were performed between one set of patient cells and one set of normal cells. In a later series there was one set of patient cells with two sets of normal cells. In addition to replicates (6 each) for

Table 9 - Mixed Leukocyte Culture in Malaria
 - One Normal Cell Population with
 One Patient Cell Population

<u>Assay #</u>	<u>Normal Cell Responders</u>	<u>Patient Cell Responders</u>
1	7.0*	2.7
2	2.2	4.9
3	1.1	3.6
4	1.1	14.5
5	1.1	34.3
6	2.3	9.1
7	1.9	31.5
8	13.6	15.6
9	6.2	9.0
10	8.6	12.8
11	12.2	22.2
12	8.0	5.1
13	2.4	2.8
14	2.5	15.4
Range:	1.1-13.6	2.7-34.3
Mean:	5.0	13.1

* Stimulation index

Table 10 Mixed Leukocyte Culture in Malaria -
Two Normal Cell Populations with
One Patient Cell Population

Assay #	Normal Responder Cells				Patient Responders	
	A + Bm	A + Cm	B + Am	B + Cm	C + Am	C + Bm
1	2.6 *					
2	3.8	1.3	3.3			
3	12.6	2.5	2.6	3.8		
4	14.1	3.7	4.4	2.7	1.8	
5	7.0	8.0	13.3	3.6	1.8	2.1
6	2.1	2.4	6.4	9.0	10.3	3.1
7	10.9	2.5	1.6	9.2	5.1	20.2
8	2.6	2.0	3.9	6.8	2.8	3.2
9	1.4	3.2	5.6	2.6	15.4	5.5
10	16.6	-	1.4	1.2	2.0	8.1
Mean:	7.0	3.4	5.3	5.9	10.0	4.0
					2.2	12.0
					1.3	38.2
					6.2	6.2
					7.0	

* Stimulation index
A = Normal cells
B = Normal cells
C = Patient cells
X_m = Mitomycin treated cells

stimulation of the patient cells by normal cells and normal cells by patient cells, controls were provided for background non-specific activity, efficacy of mitomycin C treatment and responsiveness to nonspecific stimulation (to PHA). All cell cultures were incubated at 37°C in 5% CO₂ for 5 days. The cells were then pulsed with (0.5 ug) ³H-thymidine for 24 hours. All cells were placed on filter pads in hydromix. Counts were determined in a Hewlett-Packard beta scintillation counter and stimulation indexes were determined.

RESULTS: Table 9 summarizes the results of cell mixtures of single populations of patient cells with single populations of normal cells. There developed a consistent pattern in which patient cells showed elevated response values in comparison with those of the normal cells. It was uncertain as to whether this difference was due to a true difference or due to the inability of the normal cells to respond to other cell surfaces effectively. The experiments summarized in Table ¹⁰ were performed to answer this question. Here it is seen that both normal and patient cells respond comparably and that thus foreign surface antigens of any cell type can be recognized. Therefore although the absolute numbers of circulating patient lymphocytes are reduced (as reported elsewhere), when standardized, these cells maintain the ability to recognize allogeneic markers. A manuscript on this work is in preparation. This project is complete pending redesign of experiments for more definitive investigations. This is a final report.

10. Lymphokine Mediated Macrophage Activation as Demonstrated by Enhanced Protein Synthesis

OBJECTIVE: To evaluate lymphokine mediated macrophage activation in malaria patients through the parameter of increased protein synthesis (Lowry technique).

BACKGROUND: The work of Criswell and her colleagues (17) supports the notion that humoral factors, possibly other than antibody, may activate macrophages during the malaria infection. Workers using other immunological systems have provided insight into macrophage activation (18, 19). This report summarizes data relative to macrophage activation in cases of human malaria.

METHODS: These assays were conducted according to the methodology of Nathan (20). In synthesizing lymphokines, 2 x 10⁶ patient mononuclear cells were incubated with either undiluted chimpanzee

**Table 11 Lymphokine Mediated Macrophage Activation
in Malaria - P. falciparum**

Lymphokines from Patient Cells with Malaria Antigen

<u>Patient #</u>	<u>Control</u>	<u>Test</u>	<u>Increase (ug/ml)</u>
96	62	158	96
100	129	220	91
105	82	175	93
109	40	112	72
122	101	337	236
144	80	211	131
154	90	133	42
161	160	310	150
164	175	325	151
175	242	213	(-)29
187	126	120	(-) 6
189	199	475	276
190	263	417	154
191	153	245	245

Range: (-)29-276
Mean: 126

Lymphokines from Patient Cells with Normal Red Cells

<u>Patient #</u>	<u>Control</u>	<u>Test</u>	<u>Increase (ug/ml)</u>
114	84	160	73
151	117	172	56
167	95	210	116
170	219	226	7

Range: 7-116
Mean: 63

Lymphokines from Normal Cells with Malaria Antigen

<u>Donor</u>	<u>Control</u>	<u>Test</u>	<u>Increase (ug/ml)</u>
Somchai	101	167	66
Barnyen	117	216	99
Turien	57	219	72
Prasit	177	340	162

Range: 66-163
Mean: 100

**Table 12 Lymphokine Mediated Macrophage Activation
in Malaria - P. vivax**

Lymphokines from Patient Cells with Malaria Antigens

<u>Patient #</u>	<u>Control</u>	<u>Test</u>	<u>Increase (ug/ml)</u>
102	69	317	249
117	18	111	93
125	72	111	40
123	128	149	20
129	81	194	113
131	74	132	58
133	65	292	227
135	168	199	31
139	32	82	50
141	150	206	56
148	188	213	25
150	209	247	138
156	169	321	161
158	130	179	48
184	276	365	89

Range: 31-249

Mean: 93

Lymphokines from Patient Cells with Normal Red Cells

<u>Patient #</u>	<u>Control</u>	<u>Test</u>	<u>Increase (ug/ml)</u>
155	180	148	(-)32
163	74	189	115
166	94	91	(-) 3
168	140	196	59

Range: (-)32-115

Mean: 35

Lymphokines from Normal Cells with Malaria Antigen

<u>Donor</u>	<u>Control</u>	<u>Test</u>	<u>Increase (ug/ml)</u>
Barnyen	189	303	113
Prasit	234	295	62
Katchrinnee	115	382	266
Prayote	130	257	127

Range: 62-266

Mean: 142

source falciparum antigen (WR 352 low) or with ammonium chloride extracted vivax antigen (human source) for 48 hours. Cultures were centrifuged and supernatants frozen until required. Control supernatants were prepared by incubating patient cells with media only, adding antigen immediately prior to the centrifugation step. Additional controls were culture supernatants from patient cells with normal human red cell extract or normal cells with antigen. For the adherence assay (Lowry technique) culture supernatants were incubated with normal mononuclear cells for 3 days. After decanting the supernatants, the monolayers were treated with NaOH and Lowry protein concentrations were determined.

RESULTS: Table 11 summarizes the results of 14 assays on lymphokine activity from falciparum sources with antigen. These are compared with 4 assays each for patient cells with normal red cell extract and normal white cells with malaria antigen. There resulted a mean protein value of 126 ug/ml for the patient cells with antigen compared with 100 ug/ml for the cell control and 63 ug/ml for the antigen control. These results suggest specific activity in lymphokines synthesized by the patient white cells in the presence of antigen. Table 12 displays the results of 15 assays from vivax patient cells as compared with 4 assays each with the cell and antigen control. Here the mean value for the cell control (142 ug/ml) was higher than that for the test system (93 ug/ml). This implies that where a crude extract of human antigen is used, test and control group activity may be comparable. Alternately, the enhanced results in the falciparum system could be an artifact. This assay is clearly of value in future research; however concentration with purification of malaria antigen will be necessary. Because of higher immediate priorities in other areas these assays have been discontinued. This is a final report.

11. Glucosamine (¹⁴C) Uptake in Malaria Activated Human Macrophages

OBJECTIVE: To evaluate the activation of monocytes by lymphokines synthesized by malarious lymphocytes as determined by uptake of radiolabeled (¹⁴C) glucosamine.

BACKGROUND: The mechanisms of human white cell response to malaria are poorly understood. Based on work by other investigators (19) a series of investigations were undertaken to evaluate the activation of macrophages through radioimmuno technique. This is a report of these studies.

Table 13 Glucosamine (^{14}C) Uptake in Cells Incubated with P. falciparum Antigen

Lymphocytes Present

Assay No.	Concentrated Antigen			Diluted Antigen			PHA		
	Control	Test	SI	Control	Test	SI	Control	Test	SI
1	300*	236	-	328	345	1.1	275	232	-
2	21	16	-	18	25	1.4	18	22	1.2
3	33	63	1.9	15	76	5.1	28	59	2.1
4	25	75	3.0	25	75	3.0	25	59	2.4
5	42	255	6.1	36	1050	29.1	949	455	-
6	18	94	5.2	28	47	1.7	15	124	8.3
	Mean SI+: 4.1			6.9			12.3		

Lymphocytes Absent

Assay No.	Concentrated Antigen			Diluted Antigen			PHA		
	Control	Test	SI	Control	Test	SI	Control	Test	SI
1	33	179	5.4	57	74	1.3	170	119	-
2	4	52	13	153	77	-	317	1499	4.6
3	6	10	1.6	11	18	1.6	76	298	3.9
4	121	55	-	17	83	4.9	631	522	-
	Mean SI: 6.6			2.6			4.3		

* Net counts per minute

+ Stimulation Index - negative values eliminated from calculations

Table 14 Glucosamine (^{14}C) Uptake in Cells Incubated with P. vivax Antigen

Lymphocytes Present

Assay No.	Concentrated Antigen			Diluted Antigen			PHA		
	Control	Test	SI	Control	Test	SI	Control	Test	SI
1	446	462	1.1	134	5676	42.3	1417	745	-
2	30	12	-	45	173	3.8	34	33	-
3	15	218	14.5	17	20	1.2	15	170	11.3
	Mean SI*:		7.8			15.8			11.3

Lymphocytes Absent

Assay No.	Concentrated Antigen			Diluted Antigen			PHA		
	Control	Test	SI	Control	Test	SI	Control	Test	SI
1	67	815	12.2	92	140	1.5	96	278	2.9
2	27	109	4.0	68	92	1.4	27	185	6.9
3	214	79	-	42	52	1.2	225	253	1.1
	Mean SI*:		8.1			1.4			3.6

* Negative SI's eliminated from calculations

METHODS: The assays were conducted with modifications of the methodology of Hammond and Dvorak (21). Mononuclear cells were isolated by ficollhypaque centrifugation and adjusted to a concentration of 1.5×10^6 monocytes per culture. Test antigens were either extracts of pooled P. vivax blood forms (human source) or P. falciparum (chimpanzee source). Antigen concentrations were either undiluted or diluted 1:2. Positive controls consisted of cells with the plant mitogen phytohemagglutinin. Negative controls were cells and RPMI 1640 media only. Lymphokines were synthesized either with all cells present or with lymphocytes absent. In the latter case, nonadherent cells were decanted after 2 hours and media replaced to the original volume. Lymphokines were synthesized and set up as for the previously reported (Lowry) assay. Normal cell cultures were incubated with lymphokines in 5% CO₂ for 72 hours. The cultures were next pulsed with 5 uCi of glucosamine and incubation was continued for 6 hours. Each monolayer was then washed with cold Hank's buffer solution containing non-radiolabeled glucosamine. Vials were drained and frozen at -20°C until required. Each vial was thawed and treated with cold 10% TCA with calf serum and stored at 4°C for 1 hour. After centrifugation the supernatant was decanted and hydromix added to precipitate the radiolabeled proteins. Counts were performed in a Hewlett-Packard beta counter.

RESULTS: Table B summarizes radioisotope uptake when patient cells were incubated with concentrated or diluted falciparum antigen in the presence or absence of lymphocytes. While the mean SI values for these experiments were acceptable, these results were marred by 8 negative SI values including 4 in the PHA control. Evaluation was further clouded by low CPM values-often only a few CPM's above those of background correction. Similar findings are recorded for P. vivax incubations as seen in Table 1⁴. It was concluded that the potential usefulness of this assay is dependent on concentration and purification of the malaria antigen and standardization of techniques. This assay has been discontinued in favor of those of higher priority. This is a final report.

12. Transformation of Patient Lymphocytes by Selected Malarial Antigens

OBJECTIVE: To develop methodology for the specific stimulation of malaria patient lymphocytes by erythrocyte and sporozoite antigens.

BACKGROUND: Several assays have been developed which describe specific in vitro responsiveness of host lymphocytic cells to

Table 15. Response of Malaria Patient Lymphocytes to Stimulation with
P. falciparum Antigen

(PF) Patient #	% Monocytes	Counts Per Minute	Stimulation Index
099	13	7859	2.5
104	14	3806	2.3
108	11	17021	4.6
118	10	552	5.9
130	14	2868	1.1
137	13	4037	2.9
134	9	5942	5.0
140	12	8731	3.9
142	13	10730	23.6
146	12	1735	3.1
149	10	3384	14.5
152	8	6907	24.4
Range:	8-14	552-17021	1.1-24.4
Mean:	11.6	6131	7.8

Table 16 Response of Normal Lymphocytes to Stimulation with P. falciparum Antigen
(Chimpanzee preparation) - Initial and Follow Up Values

Donor	Initial CPM / S.I.	CPM / S.I. at 24 Hours	CPM / S.I. at 1 Week
Turien (M)	7127	4319	12554
Somchai (F)	273	-	-
Prigg (M)	3699	8069	12072
Prayote (M)	8643	-	-
Prasert (M)	13399	-	-
Sanei (M)	9494	2507	9432
Prasit (M)	8689	5845	10916
Boontum (M)	14595	-	-
Komson (M)	8553	-	-
Udorn (M)	4870	-	-
Sitt (M)	4535	-	-
Kaew (M)	13687	14979	9705
Boonkum (M)	9019	-	-
Boonmee (M)	11712	-	-
Barnyen (F)	12838	-	-
Somchit (F)	247	-	-
Range:	247-14595	2507-14979	9432-12554
Mean:	8211	7144	10936
	1.9-20.0	5.4-25.2	6.2-23.9
	10.1	12.5	16.6

Table 17 Response of Lymphocytes from P. falciparum Patients to Stimulation with P. falciparum Sporozoite Antigen (1.42 ug/well)

Patient #	% Monocytes	Counts Per Minute	Stimulation Index
106	10	813	1.4
108	11	4076	1.7
134	10	2106	1.2
138	10	456	2.7
149	10	518	2.7
152	8	2494	10.1
153	14	1347	4.1
Range:	8-14	456-4076	1.2-10.1
Mean:	10.4	1687	3.4

Table 18 Response of Normal Lymphocytes to Stimulation with P. falciparum Sporozoite Antigen (1.42 ug/well)

Donor	% Monocytes	Counts Per Minute	Stimulation Index
Barnyen	12	4047	13.4
Boonmee	13	582	2.0
Somboon	11	6103	2.8
Niphon	12	6563	1.9
Sanei	10	2569	3.9
Range:	10-13	582-6563	1.9-13.4
Mean:	11.6	3973	4.8

stimulation by malaria antigens. These assays have employed either rodent (22, 23) or human material (24). This report summarizes studies utilizing related techniques, with cells from adult Thais infected with either P. falciparum or P. vivax.

METHODS: Mononuclear leukocytes were cultured in modified RPMI 1640 media according to the methodology of MacDermott et al. (25). Assays for lymphocytes from falciparum infections utilized patient lymphocytes and falciparum antigen extracted from either infected chimpanzee erythrocytes or sporozoites from human infections. Controls were uninfected cells and media. Cell suspensions were incubated 6 days at 37°C in 5% CO₂. Lymphocyte concentrations were either 3 x 10⁵ or 4 x 10⁵ cells per well with 1 ug of erythrocytic antigen or 1.42 ug of sporozoites. After initial incubation, cultures were pulsed with 0.4 uCi ³H thymidine for 24 hours. The lymphocytes were then processed by a multiple automated sample harvester (MASH). After drying filter pads containing the cells were placed in scintillation vials containing hydromix and counts (CPM) were determined in a Hewlett-Packard beta scintillation counter. Stimulation indices (SI) of patient lymphocytes were calculated by the following formula:

$$SI = \frac{CPM \text{ test}}{CPM \text{ control}}$$

RESULTS: Table 15 illustrates the mean counts and stimulation indices of twelve patients infected with P. falciparum. These results are similar to those of 9 other falciparum patients at the same cell concentration (4 x 10⁵ cells/well) under optimal conditions and to results of 9 additional falciparum patients whose cell concentration was 1 log lower under the same conditions of incubation. In comparing the results of these patients with those of normal donors (Table 16) there are no apparent differences. The range and mean values for both the counts and stimulation indices are essentially identical. Similar results were likewise observed when patient cells (Table 17) or normal cells (Table 18) were stimulated with falciparum sporozoite antigens. It is clear that definitive studies in both trophozoite and sporozoite induce transformation must await improved antigenic material through increased antigen concentration (by culture) and improved purification of human material. It is likewise imperative that the variables of parasite concentration and duration of infection be controlled to the greatest extent possible by careful patient selection in future work. Pending the constraint of these variables this research should be considered complete. This is a final report.

13. Synthesis of Lymphocyte Blastogenic Factor by Sensitized Lymphocytes from Malaria Patients

OBJECTIVE: To develop methodology for the activation of normal lymphocytes by lymphokines from antigen stimulated lymphocytes from malaria patients.

BACKGROUND: These studies were undertaken in an attempt to determine whether certain mechanisms of immune activation parallel those in better defined systems. There is considerable evidence in these systems that normal lymphocytes undergo blast transformation and incorporate ^3H thymidine when cultured in the presence of supernatants from lymphocytes stimulated with antigen (26,27). The synthesis of such blastogenic factors has been associated with T lymphocytes and is a measure of cell mediated immunity. The studies reported here represent attempts to induce blastogenesis by factors produced by specifically stimulated lymphocytes from malaria patients.

METHODS: Mononuclear leukocytes from *P. falciparum* patients were isolated by ficoll hypaque centrifugation. The cells at concentrations of either $1 \times 10^6/\text{ml}$ or $3 \times 10^6/\text{ml}$ were incubated with falciparum antigen from infected chimpanzee erythrocytes for 48 hours at 37°C in 5% CO_2 . Following centrifugation, supernatants were frozen until required. Control preparations were supernatants exposed to antigen immediately before centrifugation. For the remainder of the assay, non-sensitized (normal) lymphocytes were incubated with the supernatants for 6 days. Cultures were then pulsed with 0.4 μCi ^3H thymidine before incubating another 24 hours. The cells were isolated and washed in a multiple automated sample harvester (MASH). After drying on filter pads, the cellular material was placed in a scintillation vial with hydromix. Counts for radioactivity (CPM) were conducted on a Hewlett-Packard beta counter.

RESULTS: Table 19 summarizes the results from assays on the blastogenic activity of supernatants from 19 isolates of patient cells. The net counts for these assays ranged between 506 and 3629 (mean 1878). The stimulation indices (SI) for these counts (mean 4) met or exceeded those generally in the literature for similar studies. An attempt was made to increase the stimulation indices by increasing the concentration of lymphokine producing patient cells three fold to 3×10^6 cells/ml. The results summarized in Table 20 indicate that this attempt was not successful since both the range and mean of the SI values in six

Table 19 Blastogenesis - Lymphokines from 1×10^6 /ml
P. falciparum Lymphocytes with Normal Cells

Assay #	Mean CPM	S.I.
1	7879	1.5
2	1334	4.3
3	1405	1.3
4	506	2.1
5	912	1.9
6	1772	1.9
7	767	3.1
8	549	2.2
9	1114	5.4
10	961	4.7
11	1485	5.6
12	939	3.5
13	3629	5.9
14	3247	5.3
15	817	4.0
16	1202	5.9
17	3007	3.6
18	1988	6.8
19	2176	7.4
Range:	506-3629	1.3-7.4
Mean:	1878	4.0

Table 20 Blastogenesis - Lymphokines from 3×10^6 /ml
P. falciparum Lymphocytes with Normal Cells

Assay #	Mean CPM	S.I.
1	3332	4.9
2	2305	2.1
3	966	1.9
4	1796	3.3
5	621	1.3
6	3445	5.4
Range:	621-3445	1.3-5.4
Mean:	2076	3.2

assays were lower than when the cells were at lower concentrations. It became clear through these experiments that marked blastogenesis results from lymphokines raised by incubating patient lymphocytes with falciparum antigen. Future consideration of this assay in further immunologic analysis is therefore warranted. The evaluation of the present experimental series is, however, made difficult because of the chimpanzee origin of this antigen and by the lack of a normal chimpanzee red cell control. Definitive work with this assay must await the concentration and purification of human falciparum antigen and appropriate control material. This is a final report.

14. Continuous In vitro Cultivation of P. falciparum and Malaria Antigen Isolation

OBJECTIVE: To maintain strains of P. falciparum recovered from human subjects in a continuous in vitro culture system for large scale production of parasites.

BACKGROUND: Different techniques for in vitro culture of P. falciparum have been described (28-31). The parasites obtained were found to be infective in subsequent in vivo and in vitro experiments but the quantity produced was not sufficient for antigen preparation. Recently Trager (32) has described a continuous culture system utilizing a slow continuous flow of culture medium over a thin layer of parasitized erythrocytes. Jensen (33) has simplified the technique for propagating the viable parasites using petri dishes inside a candle jar. The system lends itself to many useful investigations including the possible preparation of a protective vaccine.

METHODS: A continuous in vitro culture system for P. falciparum was set up as described by Jensen. Infected blood specimens from malaria patient were suspended in RPMI 1640 medium supplemented with HEPES buffer and human AB serum. Target erythrocytes from individual AB blood donors were used for subcultures when parasitemias in original cultures reached about 5-6%. Aliquots of the highly parasitized cell suspension were preserved with glycerine - manitol at -70°C (34) for subsequent experiments. Different cryoprotective agents will be tested to determine the best cryopreservation method for these parasites. Further investigations are being conducted to clarify the question of the possibility of replacement of human serum in culture medium, the improvement of synchronicity of the asexual erythrocytic cycle, and infectivity after cryostorage.

RESULTS: A strain of *P. falciparum* from Sriracha, Choburi Province, has been established in a continuous in vitro culture system. This system allows an increase in parasitemia of 20 fold after 96 hours of incubation. Although individual non-immune AB blood donors were screened for optimum support in in vitro parasite growth, variations in parasite increase were observed among different donors. The use of a pooled AB serum will be attempted in subsequent cultures when adequate numbers of suitable donors are obtained in an attempt to eliminate individual differences.

Investigations are being conducted in the following areas:

1. The possibility of replacement of human serum with a more easily obtained substance in the culture medium. In the established technique, it is necessary to supplement the RPMI 1640 medium with 10% human AB serum to support in vitro development of *P. falciparum*. Preliminary results suggested that half the AB serum required may be replaced with inactivated fetal calf serum.
2. Attempts to isolate different stages of the parasite, to improve the synchronicity of the asexual erythrocytic cycle during in vitro culture, by gelatin floatation were not successful. Separation of parasites by ficoll gradient centrifugation is being evaluated.
3. Different batches of specimens frozen at -70°C using glycerol as the cryoprotective agent were tested for in vitro infectivity. The greatest loss of intact parasitized cells up to 50% was found in a specimen stored up to 60 days. Although the viability of preserved parasites in regards to the in vitro infectivity was restored, this method appeared to be unsatisfactory. Attempts will be made on other cryoprotective agents available. Storage in liquid nitrogen (-195°C) appears to be more conducive to the maintenance of intact parasitized cells. This is a preliminary report.

15. Effect of Sera from Malaria Patients on Mitogen Responsiveness of Normal Lymphocytes

OBJECTIVE: To evaluate the effect of sera from patients infected with *falciparum* or *vivax* malaria on the responsiveness of lymphocytes to stimulation with selected plant mitogens.

BACKGROUND: Stimulation of human cells with plant mitogens has been well documented (35). In this approach, nonspecific stimulation is evaluated by cellular blastogenesis or by the

Table 21 Effect of Malaria Patient Sera on Lymphocyte Response to Mitogens

	<u>Phytohemagglutinin (PHA)</u>		<u>Concanavalin A (CONA)</u>		<u>Pokeweed Mitogen (PWM)</u>	
Serum Source	<u>Normal</u>	<u>Malaria</u>	<u>Normal</u>	<u>Malaria</u>	<u>Normal</u>	<u>Malaria</u>
	138	62+	92	46	53	66
	102	69+	81	45	46	60
	102	75*	100	40	57	91
	148	64+	105	47	63	41
	99	37*	72	11	49	20
	206	49*	83	34	44	48
	181	128+	63	46	26	124
	181	81*	63	16	26	46
	191	61*	148	72	100	100
	191	45+	148	59	100	78
	101	80+	66	68	36	79
	101	87*	66	23	36	13
	151	79+	144	60	73	79
	151	111*	144	43	73	54
	190	72+	142	57	111	75
	124	47+	79	54	50	68
	131	61+	132	116	139	183
	161	105+	153	165	121	159
	192	181+	112	101	45	29
	145	59+	80	76	30	107
Range:	99-206	37-181	63-153	11-165	26-139	13-159
Mean:	149	78	104	59	65	76
<u>PF Sera Data</u> (13 Assays)						
Range:	101-192	45-181	63-152	45-165	26-139	29-182
Mean:	150	81	108	72	69	88
SD	31	38	33	35	37	44
P	0.001		0.02		0.3	
<u>PV Sera Data</u> (7 Assays)						
Range:	99-206	36-111	63-148	11-72	26-100	20-100
Mean:	147	72	97	34	55	53
SD	47	25	36	21	25	33
P	0.005		0.005		0.95	

+ P. falciparum Serum

* P. vivax Serum

BACKGROUND: While investigating the possible reappearance of malaria in the Chiangmai Valley in collaboration with the National Malaria Eradication Project from 1976 to 1977, it became apparent that potential vectors other than Anopheles minimus and Anopheles balabacensis were breeding in the study area. Because the role that these possible secondary vectors may play in human malaria transmission in Thailand is still unknown, a study to compare the susceptibility of various anopheline species to P. falciparum and P. vivax was established. Established colonies of Anopheles balabacensis (Khao Mai Khaeo strain) and Anopheles maculatus (Kuala Lumpur strain) existed at AFRIMS; thus, the susceptibility of these two species was examined first. Anopheles balabacensis is a major vector of malaria in the forested foothills of Thailand and An. maculatus is a primary vector in cleared or partially-cleared foothills in Peninsular Malaysia.

MATERIALS AND METHODS: The study was initiated in Phrabuddhabat and continued at the Kanchanaburi Provincial Hospital. Patients were admitted to the male medical ward from the hospital outpatient department or from clinics of the National Malaria Eradication Project, in Phrabuddhabat, Pak Chong and Kanchanaburi.

Sixty laboratory-reared Anopheles balabacensis and 60 Anopheles maculatus were simultaneously fed on patients prior to therapy. Ten engorged mosquitoes of both species were then withheld for determination of longevity. Mosquitoes were also fed each day on uninfected volunteers as simultaneous controls. The mosquitoes were reared and maintained either at the AFRIMS Phrabuddhabat insectary or at the insectary in Bangkok to be transported to the study site in Kanchanaburi. Both species were four to five days old on the day of feeding and were dissected seven and fourteen days post-feeding. Midguts and salivary glands were examined for oocysts and sporozoites respectively, with oocyst and sporozoite indices being determined at the time of dissection.

RESULTS: Dissection results may be summarized in two ways: individual mosquito data, and data from mosquito lots (each lot comprises 50 individuals).

Table 22 shows individual mosquito data. The percentage engorging when exposed to an infected patient was higher in the balabacensis than in the maculatus group. Also the numbers of mosquitoes surviving to dissection was higher in the balabacensis group. The positivity rate was likewise higher in balabacensis.

enhanced uptake of radiolabeled amino acids. In the present studies it was decided to search for a possible suppressive effect of pooled malaria patient sera against the responsiveness of normal lymphocytes to stimulation with phytohemagglutinin (PHA), Concanavalin A (CON A), and Pokeweed mitogen (PWM).

METHODS: Mononuclear cells were isolated by ficoll hypaque centrifugation according to the methodology of Boyum (16). The cells were washed in Selegmans balanced salt solution (SBSS) and were further processed according to the methodology of Chess et al. with modification (36). Test suspensions were made by mixing 5 ml of pooled patient (malaria species specific) sera with 20 ml of RPMI 1640 media. Similar 20% suspensions were made for control preparations in which pooled normal sera were used. Normal mononuclear cells were adjusted to concentrations of $1.5 \times 10^6/\text{ml}$ with either of the above suspensions. Mitogen suspensions were likewise made up with these sera/media mixtures to the following concentrations: PHA 50 ug/ml, CONA 20 ug/ml and PWM 250 ug/ml. A 0.1 ml volume of the respective mitogen suspension was added to each microtiter plate well along with 0.1 ml of cell suspension. Stimulation controls were comprised of cells, sera and media without mitogen. Cultures were incubated for 72 hours in 5% CO_2 and were pulsed with 0.4 uCi ^3H -thymidine for 24 hours. Cell samples were harvested onto filter paper discs and transferred to scintillation vials. After suspension in hydromix counts were performed in a Hewlett-Packard beta counter and both counts per minute and stimulation indexes were determined.

RESULTS: Table 21 summarizes the effect of patient sera on the responsiveness of normal cells to mitogenic stimulation. Significant suppression was seen where patient sera from either source was admixed. This was true regarding both responsiveness to PHA and CONA which stimulate T lymphocytes. On the other hand, significant suppression did not result in the cellular response to PWM - a mitogen which predominantly effects B lymphocytes. A manuscript is in preparation concerning this work and that involving responsiveness of patient lymphocytes to these same mitogens. This is a final report.

16. Comparative Susceptibility of Anopheles maculatus and An. balabacensis to Plasmodium vivax

OBJECTIVE: To compare the efficiency of various potential vectors of P. vivax. To evaluate the susceptibility of "secondary vectors".

TABLE 22

FEEDING AND DISSECTION RESULTS FROM MOSQUITOES
FED ON UNTREATED P. VIVAX PATIENTS

	<u>Anopheles</u> <u>balabacensis</u>	<u>Anopheles</u> <u>maculatus</u>
Total number of mosquitoes fed (% engorged)	5283 (80)	4145 (63)
Total number dissected	3403	1935
Percent survival	82	74
Total number dissected (oocysts) (% positive)	743 (27)	591 (19)
Total number dissected (sporozoites) (% positive)	2660 (22)	1326 (16)

TABLE 23

DISSECTION RESULTS FROM MOSQUITO LOTS
FED ON UNTREATED P. VIVAX PATIENTS

	<u>Anopheles</u> <u>balabacensis</u>	<u>Anopheles</u> <u>maculatus</u>
Number of mosquito feeds	109	109
Number of feeds positive (oocysts)	40	33
Number of feeds positive (sporozoites)	44	33
Total number of feeds positive (% positive)	49 (45)	39 (36)

TABLE 24

RESULTS OF DISSECTION OF 14 LOTS OF
Anopheles balabacensis AND An. maculatus
POSITIVE FOR PLASMODIUM VIVAX

	<u>Anopheles</u> <u>balabacensis</u>	<u>Anopheles</u> <u>maculatus</u>
Mean # of oocysts/gut (range)	30.5 (2-110)	19.2 (2-86)
Mean diameter of oocysts (range)	43.9 (30-60.4)	41.8 (24-58.4)
Mean sporozoite level (range)	+3.1 (+2 - +4)	+2.6 (0 - +4)

Table 23 presents mosquito lot data. Again, lots of balabacensis were more likely to be infected than maculatus, and the positivity rate was higher among balabacensis lots.

When positive lots were compared in terms of oocyst numbers/gut, oocyst diameters, and sporozoite densities (Table 24) vivax oocysts were found to be greater in number and of larger mean diameter in balabacensis than in maculatus. Sporozoites were found in glands in greater numbers in balabacensis than in maculatus.

Generally, laboratory-reared An. balabacensis appear to be more efficient vectors than laboratory-reared An. maculatus, in terms of engorgement, survival, positivity, and numbers of oocysts and sporozoites developing in the mosquito.

When suitable colonies of other potential vectors are established at the Laboratory, it is planned that they will be tested in a similar fashion.

17. Longevity Studies on Malaria Infected Anopheles

OBJECTIVE: To test the effects of human malaria parasites on the longevity of the host mosquito.

BACKGROUND: Most previous investigations on the effect of malaria parasites on the mosquito host have been inconclusive. De Buck and Swellengrebel (37) found greater mortality among heavily infected mosquitoes in relation to lightly infected ones. However, Boyd (38) and Ragab (39) concluded that malaria parasites have no detrimental effects on the longevity and vitality of the host mosquito. More recently, Schiefer et al (40) found that reductions in the flight capabilities of infected Anopheles stephensi mosquitoes were positively correlated to the severity of Plasmodium cynomolgi infection. During the past several years AFRIMS investigators, collaborating on malaria drug testing in the rhesus monkey model, have noted a detrimental effect on mosquitoes infected with P. cynomolgi. Procedures during this study called for the production of maximum numbers of sporozoites in the laboratory vector, An. balabacensis.

Observations indicated that high oocyst numbers and subsequently high sporozoite numbers led to increased mortality in the host mosquitoes. The highest mortality was observed on day 2 and day 12 post-feeding, probably corresponding with the initial time of invasion of the midgut by ookinetes and later the rupturing

TABLE 25 Comparison of longevity in Plasmodium vivax-infected and uninfected anopheline mosquitoes

	<u>Anopheles balabacensis</u>		<u>Anopheles maculatus</u>	
	Infected	Uninfected	Infected	Uninfected
Number of lots	22	19	20	13
Longevity in days				
Mean	21.3	23.5	16.9	21.0
Range	1-38	1-51	2-34	2-34

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of the oocysts on the midgut and the invasion of the salivary glands by sporozoites of P. cynomolgi. In order to determine whether increased mortality occurred in anopheline mosquitoes infected with human malaria, a study was designed to compare the longevity of uninfected mosquitoes with those infected with P. vivax. A reduction in vector longevity could be important information in the study of the epidemiology of human malaria.

METHODS: Sixty laboratory-reared Anopheles balabacensis (Thai strain) and 60 An. maculatus (IMR strain) were fed on human malaria patients in Phrabuddhabat or Kanchanaburi on the day of hospital admission and days 1, 7, 14, and 21 of follow-up. Ten engorged mosquitoes of each species were then withheld for longevity studies and the remainder were used for dissections and other experiments. Mosquitoes were also fed each day on uninfected volunteers as a simultaneous control. The mosquitoes had been reared and maintained either at the AFRIMS Phrabuddhabat insectary, or at the Bangkok insectary to be transported to the study site in Kanchanaburi. Females of both species were 4 to 5 days old on the day of feeding and were dissected 7 and 14 days post-feeding. Midguts and salivary glands were examined for oocysts and sporozoites respectively, with oocyst and sporozoite indices being determined at the time of dissection. Infected and control specimens were maintained as the original lot (10 specimens) in small screened paper cups supplemented daily with a multivitamin syrup solution for nutrition. All cups were kept in the same room, so that both infected and control mosquitoes were exposed to the same temperature and humidity.

RESULTS: A total of 22 infected lots and 19 uninfected were compared for balabacensis, and 20 infected lots and 13 uninfected lots for maculatus. The life span for both infected and uninfected balabacensis females ranged from 1-51 days, while that for maculatus ranged from 2-34 days. Mean survival days for both infected and uninfected females of both species are shown in Table 25. For both balabacensis and maculatus, uninfected females lived longer than infected females, indicating that malaria infections in mosquitoes may have a detrimental effect on the vector. Infected females of balabacensis lived longer than uninfected maculatus. The longer life span of balabacensis might be partially responsible for this species being such an efficient vector in nature.

These studies have been completed.

Project 3M62770A803 MALARIA PROPHYLAXIS

Work Unit 089 Field studies on drug resistant malaria (AFRIMS)

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<p>23. (U) The technical objectives of this work are (1) to develop analytical methods for analysis of nerve agent antidote starting materials and final product (2) to provide in-process and final product analytical control for production of nerve agent antidote. (3) to evaluate stability of formulated nerve agent antidote for establishing shelf life. (4) to elucidate the cause and mechanisms of degradation/alteration of components in formulation and develop modes of stabilization. (5) to provide reference standards to assure quality to procured materials for nerve agent antidote. These investigations are designed for perfection on prophylaxis against possible combat casualties by nerve agents.</p> <p>24. (U) The objectives will be met by applying UV spectroscopy, gas liquid chromatography, mass spectrometry, thin layer chromatography, high performance liquid chromatography and other standard analytical techniques for analysis of starting materials and the formulations under investigations for various experimentation and the final product.</p> <p>25. (U) 77 10 - 78 09 Gas liquid chromatographic and UV spectrophotometric methods were established and high performance liquid chromatographic developed for the analysis of the nerve agent antidote components, impurities and degradation products. High performance liquid proved to be superior for these analysis. Reference standards were prepared and quality control maintained. The stability of the antidote was studied under various storage conditions. Based on the time required for Benactyzine to reach 75 percent of the original concentration, the antidote was found to be stable for over 10 years at 5 degrees C. At 25 degrees, 40 degrees, and 54 degrees C, Atropine Sulfate and TMB-4 were stable; Benactyzine deterioration was a function of temperature and followed a zero order reaction rate. This work was terminated by administrative action. For technical report see WRAIR Annual Progress Report 1 Oct 77 to 30 Sep 78.</p>						

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Project 3M162770A803 MALARIA PROPHYLAXIS

Work Unit 091 Analytical Support for Nerve Agent Antidote Production

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The objectives of this work unit are to develop and utilize methods for determining the stability of the components of nerve agent antidote under various conditions of storage, to ascertain the shelflife, and to recommend storage and shipping conditions. In the interest of assuring a good quality product at all times and at the same time to minimize the logistical cost of storage and replacement of outdated material, it is essential that the mechanism of deterioration of the nerve agent antidote components be understood and that the influence of various environmental factors on these mechanism be fully evaluated. The following studies were conducted.

1. The stability of components of the nerve agent antidote under various storage conditions.
2. The identification of a plasticizer found in nerve agent antidote injectors.
3. The quantification of components in the nerve agent antidote following ambient storage.

1. The stability of components of the nerve agent antidote under various conditions involving time and temperature.

Nerve agent antidote injectors from a single lot obtained from Defense Personnel Support Center are subjected to various storage conditions for specific time periods. Replicate samples are analyzed to quantify precisely the levels of the active ingredients, preservatives, breakdown products, and impurities to establish a total product balance for each storage condition and storage time period. The injectors are grouped as indicated in Table I.

Group I serves as the standard against which all analyses are compared.

Group II provides the data to define stability of the antidote in refrigerated storage.

Group III provides the data to define stability of the antidote at room temperature.

Group IV and V provide the data to define the stability of the antidote at elevated temperatures which may be encountered under operational conditions.

Group VI provides the data to test the hypothesis that storage at low temperature does not decrease solubility of the components and therefore reduce the delivered dose and that freezing does not effect the stability of the product.

Groups VII, VIII, IX and X provide data to test the hypothesis of the ingredients of the antidote are catalytic for the breakdown once it is initiated. These data provide the basis for estimating the effect of short intervals of elevated temperature on the stability of the antidote.

Group XI provides data to test the hypothesis that storage at 5°C following exposure to 54°C will prolong the stability of the components.

Samples are prepared by extruding the contents of the Injector into individual siliconized, stoppered glass vials. Portions were removed for the individual analysis. TMB-4-Br was analyzed by ultraviolet spectrophotometry (UV) and High Performance Liquid Chromatography (HPLC); Benactyzine by Gas-liquid Chromatography (GLC) and HPLC; Atropine by GLC; Methylparaben and Propylparaben by HPLC (WRAIR Progress Report FY 77-78). Data are analyzed by regression analysis to estimate the order of the reaction and the rate constant. Regression and location parameter estimates are defined at 95% confidence levels using an assumed distribution for these parameter estimates.

The lot (CTS-3-2) of TAB injectors was formulated in May 1976, shipped to Division of Biochemistry, WRAIR, on Feb 1977, and stored at 5°C. The stability study was started 30 June 1977. The initial analysis of injectors showed that some degradation of TAB components had begun between the time of preparation of injectors and the initial analysis. The results of these initial analysis are reported as Group I and are used as a baseline for the stability studies. Data are included for Group I through XI.

The analytical data for Group I (Baseline values) are showed in Table 2.

The low concentrations of propylparaben found in Group I can be accounted for in part (15 to 20%) by its association with the Butyl rubber septums and by the presence of P-hydroxy benzoic acid (hydrolysis product of the parabens) in solution and associated with

the septums.

A. Atropine Sulfate:

Atropine sulfate, analyzed by GLC, was shown to be stable under experimental conditions (Table 3).

B. TMB 4. BR:

TMB-4. Br, analyzed by UV spectroscopy was shown to be stable under studies experimental conditions (Table 3). However, when the analysis were carried out by HPLC method and the data from control group I was compared with data from experimental groups IV, V, and XI, these groups showed a decrease in concentration. A linear regression analysis of the data for Groups II and V is shown in Figure 1. The predicted time for Group II and V to decrease to 90% of Label would be 10.6 years and 1.2 years respectively.

C. Methyl Paraben:

Methylparaben, analyzed by HPLS, was significantly decreased in studied experimental groups when compared to Group I (Table 4). The decrease in Methylparaben concentration was related to time (Group II) and well as temperature (Table 4 and Figure 2).

Some Methylparaben, 5 to 10% of labeled concentration, was found associated with the butyl rubber septums of the cartridges. The hydrolysis product of the parabens, p-hydroxy benzoic acid, was increased from about 5% in Group I to 15 to 20% in Groups IV and V which indicated that the hydrolysis of the parabens was temperature dependent.

D. Propylparaben:

Propylparaben, analyzed by HPLC, was decreased significantly in Group III, IV, V, and XI when compared to Group I (Table 5). The decrease in propylparaben concentration was also a function of both time and temperature (Figure 3).

About 5 to 10% of labelled concentration of propylparaben was associated with the butyl rubber septums of the cartridge. The low concentration of Propylparaben found in Group I cannot be accounted for entirely at this time.

E. Benactyzine. HCl:

Benactyzine . HCl, analyzed by GLC, was significantly decreased in Groups IV, V, and XI when compared to Group I (Table 6). The decrease in Benactyzine HCl concentration was temperature dependent (Table 6 and Figure 4).

The predicted times to reach 75% of initial concentration of Benactyzine (Table 7) are based on a linear regression analysis (Figures 4, 5 and 6).

F. Stability of TAB Components Under Other Storage Conditions

1. Freezing the TAB solution for two weeks and then storing for 4 months at 25°C (Groups VI) had no effect on the stability of Atropine sulfate, Benactyzine. HCl, or TMB-4. Br. The methylparaben concentration was significantly decreased but this was a function of time more than storage conditions.

2. Heating the TAB solution for two weeks and at 40°C then storing for 4 months at 25°C (Group VII) had no effect on the stability of Atropine Sulfate, Benactyzine. HCl, or TMB-4 Br. The methylparaben concentration was significantly decreased but this was a function of time more than storage conditions.

3. Heating the TAB solution for two weeks at 54°C then storing for 4 months at 25°C (Group VIII) had no effect on the Atropine Sulfate or TMB-4. Br. However, Benactyzine. HCl concentration was significantly decreased over baseline levels, but the decrease occurred during the initial 2 weeks at 54°C. The values immediately after heating and for the next 4 months were 3.56, 3.42, 3.74, 3.55, and 3.63 mg/2ml respectively. The methylparaben concentration was significantly decreased but was mostly a function of time rather than storage conditions.

4. Heating the TAB solution for two weeks at either 40°C and then storage at 5°C for one year (Groups IX and X) had no effect on Atropine Sulfate or TMB-4, and the results for Benactyzine, Methylparaben and propylparaben were similar to groups VII and VIII.

5. Group XI samples of TAB which were alternately heated (54°C) and cooled (5°C) for two weeks periods, showed that refrigeration after heating does extend the storage time of the TAB components. This observation is supported by the Group VIII studies.

Discussion:

The stability of the ingredients of TAB nerve agent antidote was assessed under various conditions of storage and time for establishing the shelf life, storage and shipping conditions of the product. The results obtained from the present study support the hypothesis that the active components of the TAB nerve agent antidote are stable at refrigerated temperature (5°C) and that the rate of Benactyzine degradation and also to a lesser extent TMB-4 are temperature dependent. In addition, the degradation of Methylparaben and propylparaben is a function of both the time and temperature. Atropine sulfate was found to be a stable under all experimental conditions employed in

these studies.

The study shows that the stability of antidote during storage, shipping or field condition depends on the length of time and temperature to which it is exposed. A partial answer to the question, how long can the antidote solution be stored or taken out of refrigeration without significantly affecting the shelf life of the product can be obtained from the present studies by making certain stipulations. For example, the time required for Benactyzine HCL, the most labile active component, to decrease to 75% of its initial concentration (Table 7) would be over 10 years at 5°C., 2 to 2.5 years at 25°C and only months at 40°C or 54°C. These figures are in general agreement with Zvrblis and Ellin except in case of 25°C. They estimated the time at 25°C to be 1.6 years. The presumed stipulation regarding a Benactyzine HCl concentration of 75% of the initial concentration as a reference point for predicting shelf life of TAB was based on studies reported by the Biomedical laboratory at Edgewood MD (TAB-IND). Benzactyzine thermally degraded to about 75% of initial concentration was shown to be as effective against GD in the mouse and rabbit. The ratio of Atropine sulfate, TMB-4 and Benactyzine to be effective against GD in species such as in mouse, rabbit, monkey and human are different. Also, the toxicity of these components are different in each species. This being the case the 75% value of benactyzine in TAB formulation should be considered as an arbitrary figure until systematic bioefficacy and safety studies are conducted using TAB formulation containing various concentrations of Benactyzine 0-100% at the variation of 10-15% levels) using several species of animal. The results thus generated, if correlated with the results from the present study, would firmly establish the useful shelf life of the formulation under various conditions. Other related questions that can be answered from the present studies are: (a) that the time TAB was subjected to an elevated temperature could be substrated from the total shelf life (Group VIII), (b) the storage at refrigerated temperatures following exposure to an elevated temperature prolonged the degradation of TAB components, it was observed that the thermal degradation of Benactyzine in TAB injectors after initial 4 weeks at 54°C followed zero order reaction. This reflected the condition after the degradation had been initiated. Benzoic acid was found as a degradation product but quantitatively could not account for the total Benactyzine breakdown. Therefore it is possible that, either Benzoic acid was further degraded to other product (s), or the methodology to identify and characterize all the degradation product (s) of this reaction is not yet perfected. The degradation of parabens posed the same analytical problem in that the levels of hydroxybenzoic acid could not be equated to the hydrolysis of the parabens. (d) Parabens can be analyzed using HPLC method and (e) the desirability for a multiple methods utilization in the analysis of the components of the TAB formulation is quite evident when one

compares the results of UV vs HPLC analysis of TMB-4.

The most promising of the analytical systems for the study of the TAB components continues to be HPLC. Recently with further modifications in the system, it has been possible not only to resolve and quantitate all components of the TAB formulation, but also many of the impurities and degradation products.

Based upon present information, the following investigations should be carried out in order to firmly establish the useful shelf life, obtain approval for human use by FDA and if possible, improve the stability of TAB formulations.

1. The identification and quantitation of the impurities and degradation products of the TAB formulation, including the mechanism and Kinetics of degradation of Benactyzine (IND Requirements).
2. If the determination of the shelf life and safety of the TAB formulation is to be based on the stability of Benactyzine HCl, the least stable component, then the studies should be carried out to determine the safety and bioefficacy of the TAB formulation containing concentration of Benactyzine (thermal degraded) ranging from 0-100% of the initial concentration. This information in conjunction with thermal and time stability study result will be useful in firmly establishing the shelf life and safety.
3. TAB injectors currently in storage should be stored either under refrigerated condition or if that is not possible, they should be stored at 25° with + 2°C variation. In our experience any short term exposure to elevated temperatures will result in a proportionate amount of degradation of Benactyzine. These injectors should be analyzed periodically to assure the quality and safety of the product.
4. Attempts should be made to stabilize the formulation. One possible approach could be the substitution of buffer at appropriate pH in place of HCl in formulation.
5. An ingredient (plasticizer) is leached from the butyl rubber septum used in the injector. The identification, physiological effects of this component and its possible effects on the stability of TAB components may become essential at some stage on the investigation.
6. Consideration should be given to modify the physical structure of the injector and TAB components in order to eliminate the degradation of formulation.

Table I

Grouping of Injectors for Stability Studies

<u>Group</u>	<u>Nr</u>	<u>Treatment</u>
I	10	Immediate assay to verify quality of lot CTX-3-2.
II	200	Storage at 5°C, analysis of 3 injectors per week x 25 weeks; analysis of 3 injectors per month for duration of study.
III	200	Storage at 25°C, analysis of 3 injectors per week x 25 weeks; analysis of 3 injectors per month for duration of study.
IV	111	Storage at 40°C, analysis of 3 injectors per week x 25 weeks; analysis of 3 injectors per month for 12 additional months.
V	75	Storage at 54°C, analysis of 3 injectors per week x 25 week.
IV	15	Storage at - 20°C for two weeks, analysis of 3 injectors, storage at 25°C for an additional 4 months, analysis of 3 injectors per month.
VII	15	Storage at 40°C for two weeks, analysis of 3 injectors, storage at 25°C for an additional 4 months, analysis of 3 injectors per month.
VIII	15	Storage at 54°C for two weeks, analysis of 3 injectors, storage at 25°C for an additional 4 months, analysis of 3 injectors per month.

IX	15	Storage at 40°C for two weeks, storage at 5°C, analysis of 3 injectors every third month.
X	15	Storage at 54°C for two weeks, storage at 5°C, analysis of 3 injectors every third month.
XI	36	Storage at 54°C for two weeks, analysis 3 injectors; storage at 5°C for two weeks, analysis 3 injectors. Alternate storage at 54°C and 5°C for two week intervals analyzing 3 injectors and heating and cooling cycle for 6 months.

Table 2

Analytical Data For Tab Samples In Group 1

Compound	Method of Analysis	Concentration mg/2ml+ S.D.	Percent of Label	95% Confidence Limits	%Coefficient of Variation
Atropine Sulfate	GLC	1.006 \pm .008	103.5	1.047 - 1.085	0.8
Benactyzine. HCl	GLC	3.89 \pm 0.11	94.0	3.55 - 4.23	2.9
Benactyzine. HCl	HPLC	3.92 \pm 0.14	94.7	3.58 - 4.24	3.8
TMB - 4.Br	UV	40.80 \pm 0.44	104.0	39.89 -41.72	1.1
TMB - 4. Br	HPLC	40.70 \pm 1.32	103.7	37.76 -43.72	3.2
Methylparaben	HPLC	0.897 \pm .029	89.7	0.831 - 0.963	3.3
Propylparaben	HPLC	0.0488 \pm .0030	48.8	0.0405- 0.0571	7.6

Table 3

Analysis of TAB formulation for Atropine Sulfate and TMB-4. Br.

Group	Atropine Sulfate mg/2ml+ S.D. GLC method	TMB-4. Br. mg/2ml+ S.D. UV method	TMB-4. Br. mg/2ml+ S.D. HPLC method
II	1.055±0.020	40.76±0.56	40.27±0.89
III	1.057±0.020	40.62±0.46	40.22±0.83
IV	1.068±0.025	40.57±0.64	39.35±1.04**
V	1.061±0.032	40.40±0.64	38.85±1.30**
IV	1.037±0.004	40.45±0.29	40.65±0.52
VII	1.041±0.020	40.17±0.50	40.24±0.59
VIII	1.044±0.023	40.17±0.48	40.36±0.99
XI	1.053±0.001	40.58±0.04	39.19±1.62**

The data represents the average of all analysis performed for 26 weeks as described in text for each group.

**p<0.01 C.W. Dunnett, A multiple comparisons test for comparing several treatments with a control. Institute of Mathematical Statistics Meeting, Ithaca, New York, 1954.

Table 4

Methylparaben concentrations of Tab Samples in Groups I-V and XI with Time

Time Weeks	<u>Groups</u>					
	I mg/2ml %	II mg/2ml %	III mg/2ml %	IV mg/2ml %	V mg/2ml %	XI mg/2ml %
0	0.897 100					
4		0.870 97	0.883 98	0.840 94	0.800 89	0.827 92
8		0.880 98	0.870 97	0.820 91	0.780 87	0.790 88
12		0.880 98	0.850 95	0.810 90	0.690 77	0.780 87
16		0.870 97	0.830 92	0.770 86	0.670 75	0.760 85
20		0.850 95	0.810 90	0.750 84	0.610 68	0.700 78
24		0.830 92	0.790 88	0.680 76	0.570 64	0.680 76
26		0.800 89	0.760 85	0.645 72	0.530 59	

Table 5
Propylparaben Concentrations of TAB Samples In Groups I-V
And XI With Time

Time Weeks	<u>Groups</u>									
	I	II	III	IV	V	XI				
	mg/2ml	mg/2ml	mg/2ml	mg/2ml	mg/2ml	mg/2ml	%	%	%	%
0	0.049	100	0.045	0.040	0.035	0.040	82	71	82	82
4										
8		0.048	0.045	0.039	0.032	0.035	80	65	71	71
12		0.052	0.041	0.034	0.029	0.028	69	59	57	57
16		0.048	0.037	0.024	0.023	0.021	49	47	43	43
20		0.044	0.032	0.022	0.017	0.016	45	35	33	33
24		0.040	0.036	0.017	0.016	0.018	35	33	37	37
26		0.035	0.029	0.018	0.011		37	22		

Table 6

Groups

Table 7

Time for Benactyzine HCl Concentration to Decrease to
75 Percent of Initial Concentration

Group/Temp C	Predicted Time
II - 5	<10 yrs
III - 25	2.0 - 2.5 yrs
IV - 40	4.0 - 4.5 mo
V - 54	1.3 - 1.6 mo
XI - 54/5	2.0 - 2.4 mo

Figure 1
TMB-4 by HPLC

$$y = A + Bx$$

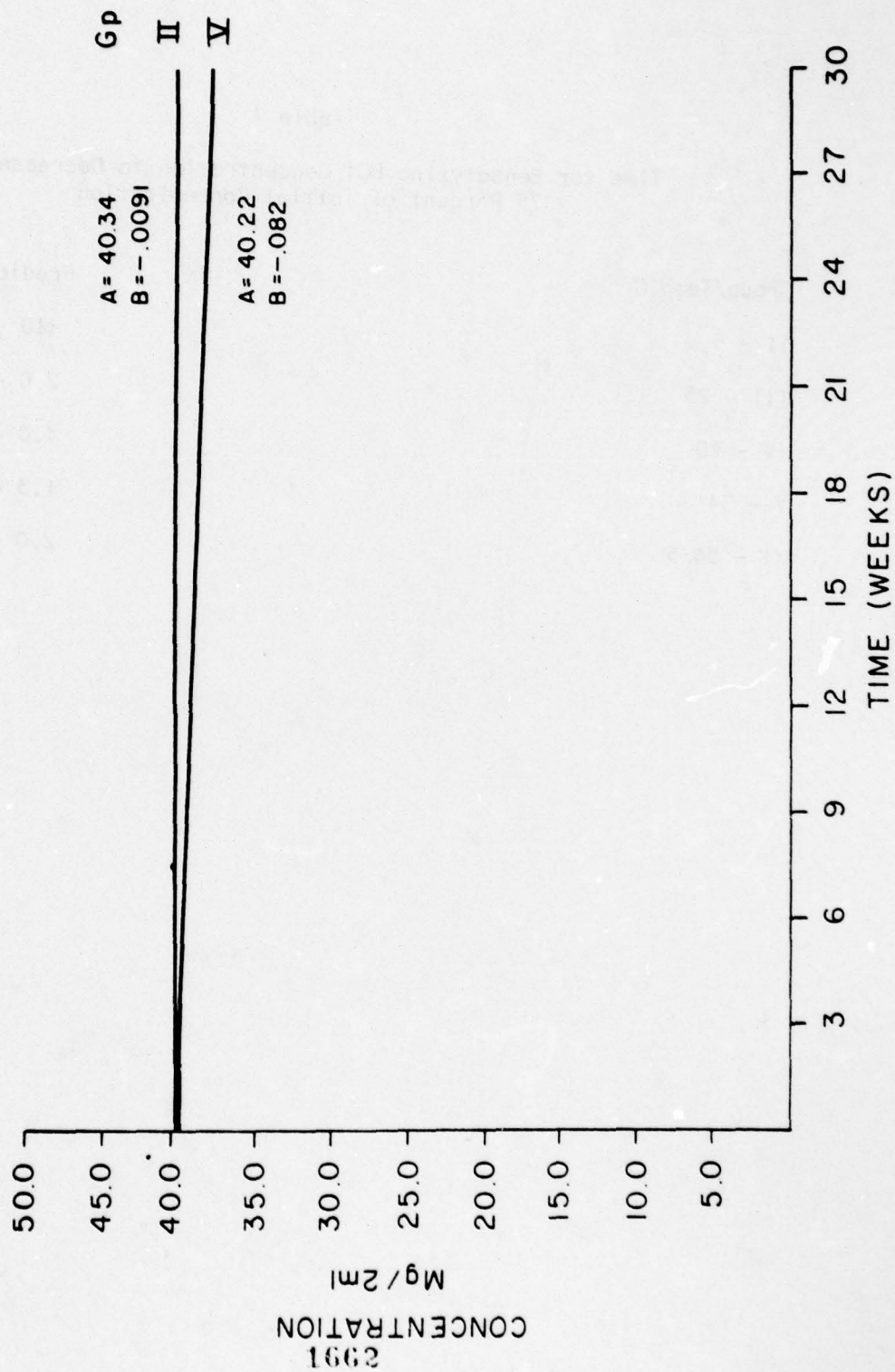


Figure 2
Methylparaben by HPLC

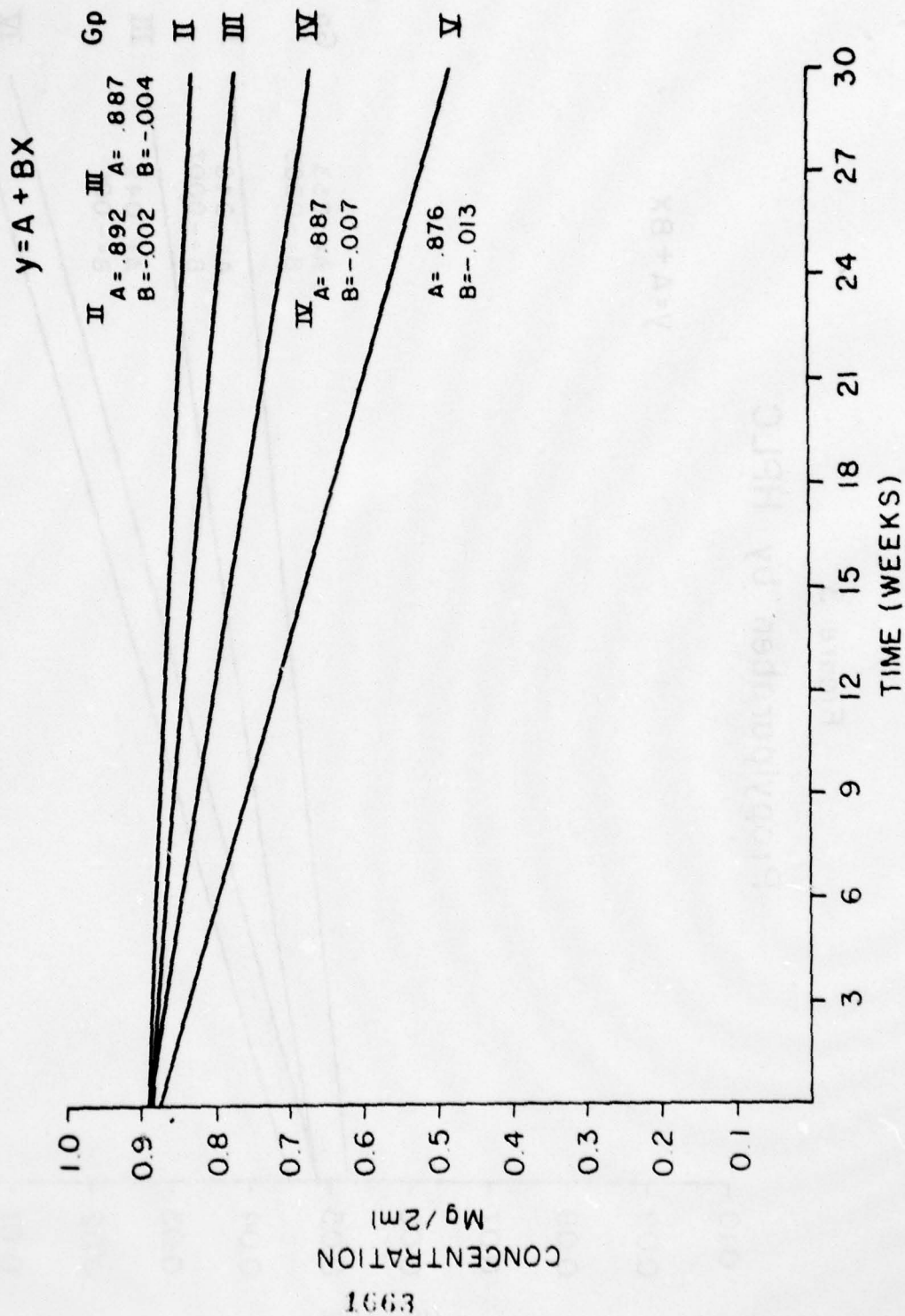


Figure 3

Propylparaben by HPLC

$$y = A + BX$$

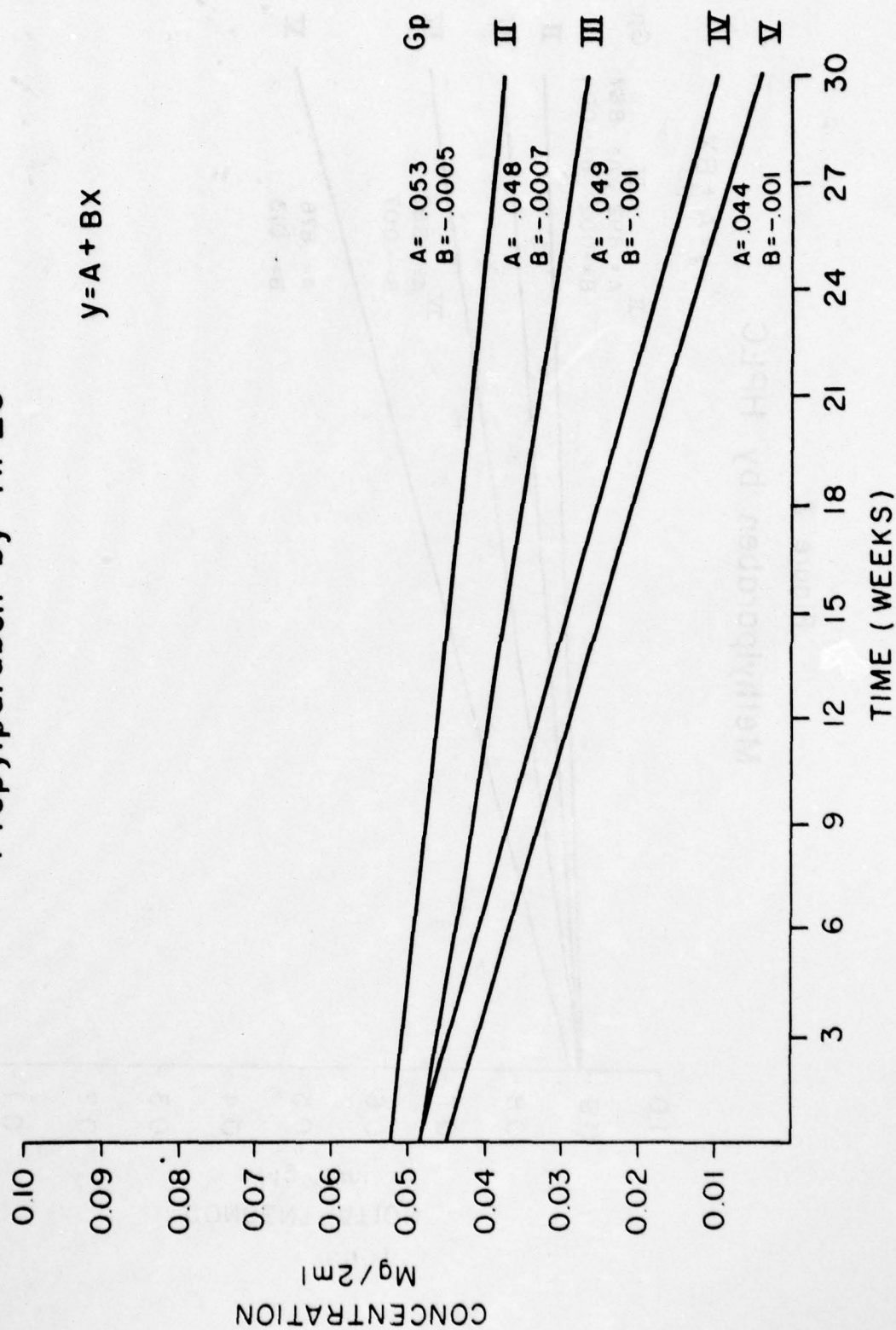


Figure 4
Benactyzine by GLC

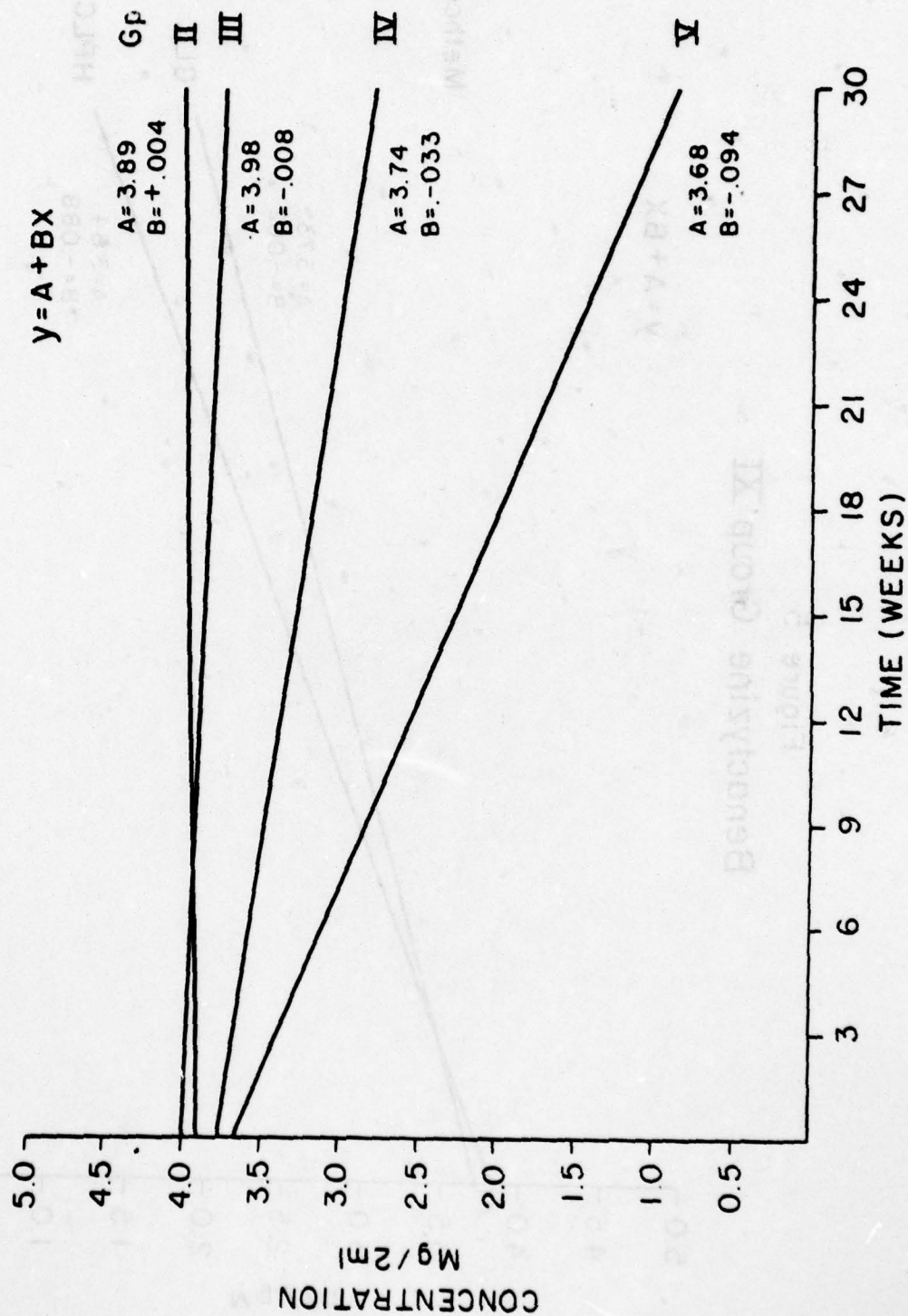


Figure 5
Benactyzine Group XI

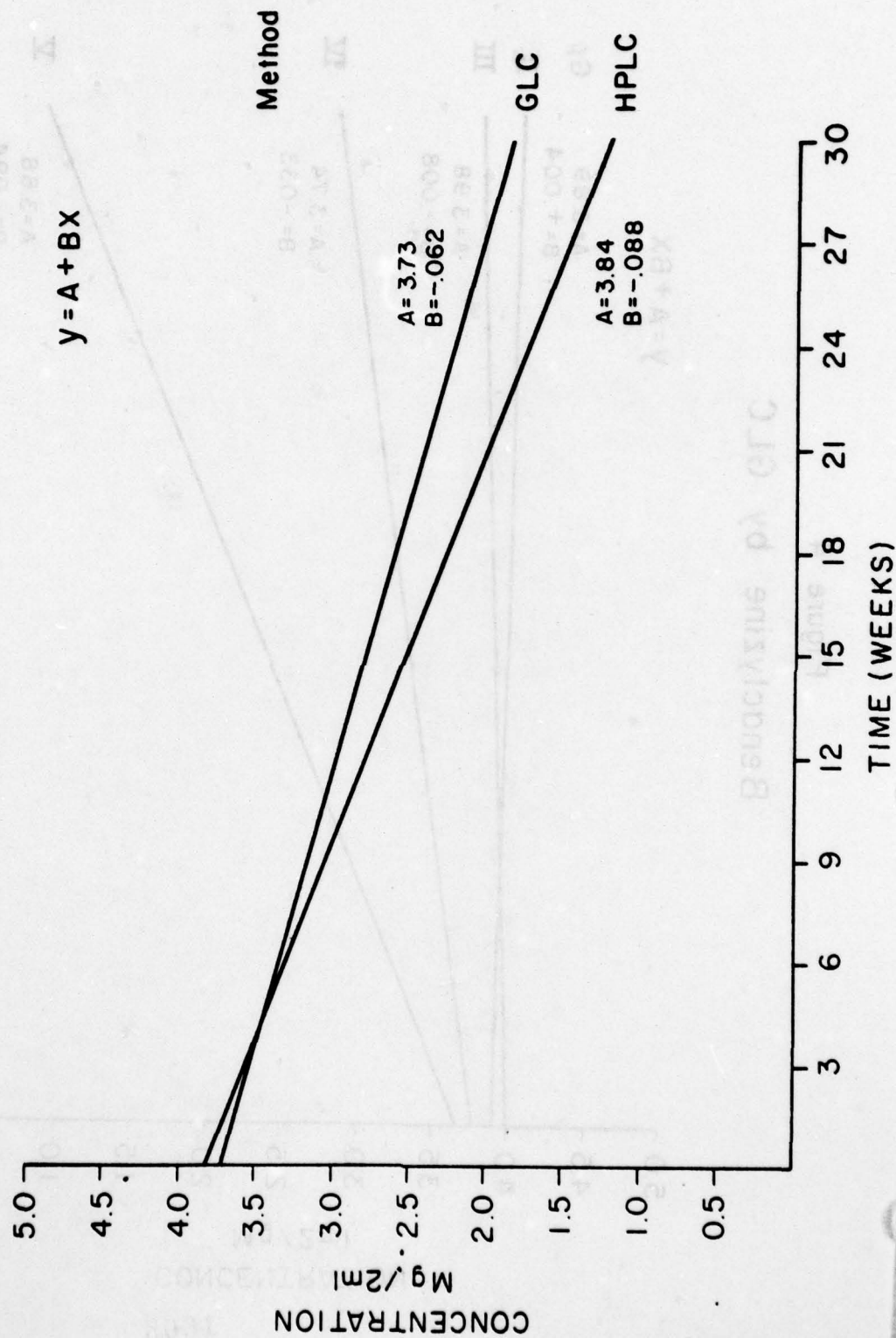
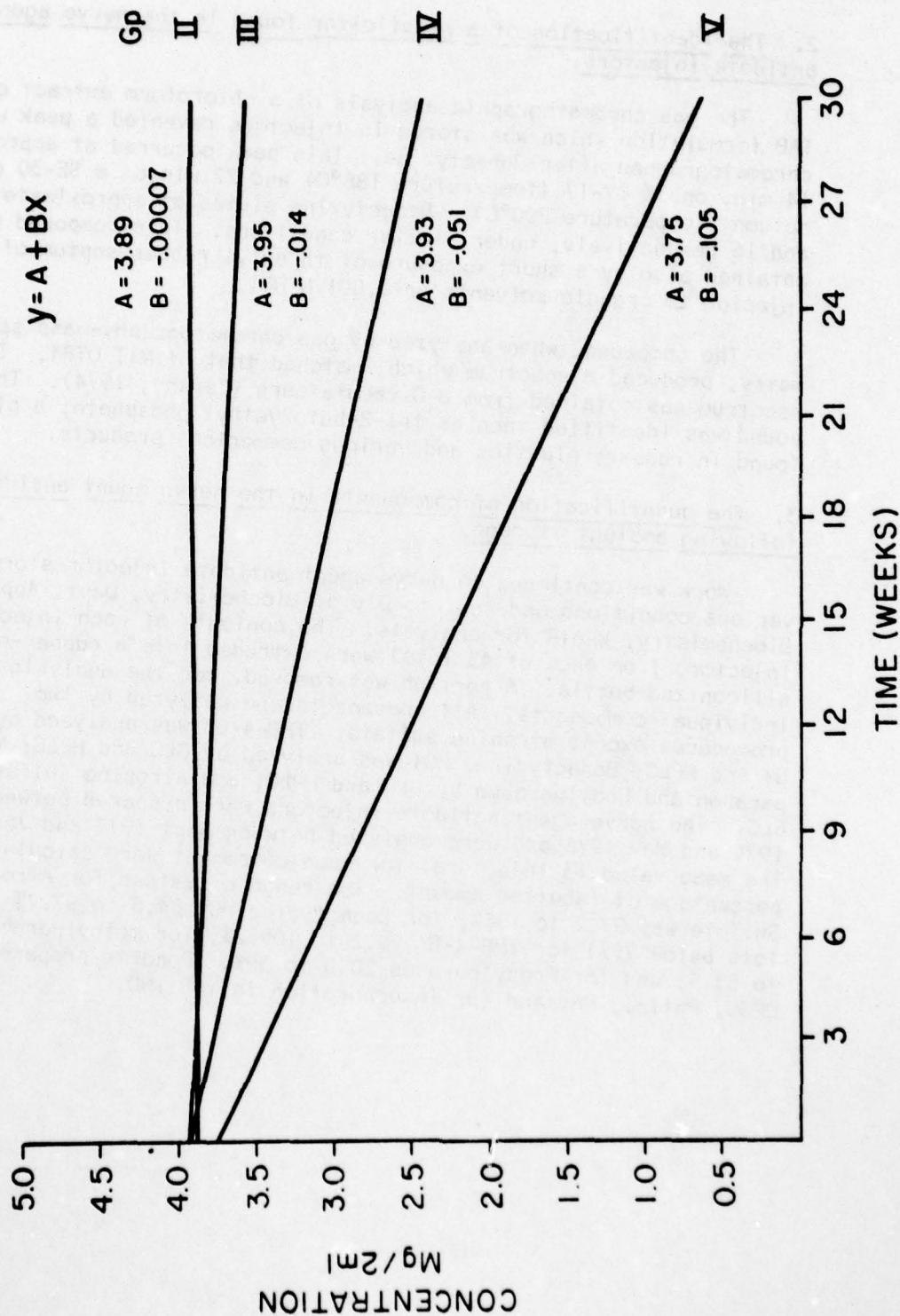


Figure 6
Benactyzine by HPLC



2. The Identification of a plasticizer found in the nerve agent antidote injectors.

The gas chromatographic analysis of a chloroform extract of the TAB formulation which was stored in injectors revealed a peak which chromatographed after Benactyzine. This peak occurred at approximately 14 min. on 3% OV-17 (temperature 188°C) and 22 min on a SE-30 capillary column (Temperature 200°C). Benactyzine eluted at approximately 8 min and 16 respectively, under similar conditions. This compound was obtained also by a short exposure of the butyl rubber septum of the injector to organic solvents or 0.001 N HCl.

The compound, when analyzed by gas chromatography-mass spectrometry, produced a spectrum which, matched that of MIT 0784. This spectrum was obtained from B-D vacutainers (Farshy, 1974). The compound was identified then as tri-2-butoxyethyl phosphate, a plasticizer found in rubber, plastics and various commercial products.

3. The quantification of components in the nerve agent antidote following ambient storage.

Work was continued on nerve agent antidote injector stored under various conditions and sent to Div of Biochemistry, Dept. Applied Biochemistry, WRAIR for analysis. The contents of each injector (3 injectors from each of 43 lots) were extruded into a rubber-stoppered, siliconized bottle. A portion was removed for the analysis of the individual components. All components are analyzed by two procedures except atropine sulfate: TMB-4-Br was analyzed by UV and HPLC; Benactyzine HCl was analyzed by GLC and HPLC; Methylparaben and Propylparaben by GLC and HPLC; and Atropine Sulfate by GLC. The nerve agent antidote injectors were prepared between Sept 1975 and May 1976 and were analyzed between Sept 1977 and Jan 1978. The mean value (3 injectors) for each component were calculated as percentage of labelled amount. The range of values for Atropine Sulfate was 97.2 to 106%; for Benactyzine HCl 54.6 to 97.7% (9% of lots below 75%) for TMB-4-Br 99.2 to 105.2%, for methylparaben 65.0 to 83.%; and for Propylparaben 20.0 to 39%. Reports prepared for DPSC, Phila., PA. and for incorporation in TAB IND.

Literature Cited.

References:

1. Farshy, D.C.: Tri-butoxyethyl phosphate as a contaminant in B-D Vacutainers. *Applied Microbiology* 27: 300, 1974.

Publications:

1. Brown, N.D. Hall, L.L., Sleeman, H.K., Doctor, B.P. and Demaree, G.E.: Ion-Pair High Performance Liquid Chromatography Separation of a Multicomponent anticholinergic Drug Formulation. *J. Chromatography* 148:453-457, 1978.

2. Brown, N.B. and Sleeman, H.K.: Ion-Pair High Performance Liquid Chromatographic method for the Determination of Atropine Sulfate and Tropic Acid. *J. Chromatography* 150:225-228, 1978.

3. Sleeman, H.K., Brown, N.D., Doctor, B.P. and Demaree, G.E.: Stability Studies on a Multicomponent Nerve Agent Antidote. Annual Meeting American Chemical Society, Abstract, 1978.

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Project 3E162771A804

MILITARY PSYCHIATRY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL ^a	
				DA OC 6450	78 10 01	DD-DR&E(AR)636	
3. DATE PREV. SUPPLY	4. KIND OF SUMMARY	5. SUMMARY ACTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DESIG. INSTR ^a	9. SPECIFIC DATA - CONTRACTOR ACCESS ^a	10. LEVEL OF SUM ^a
77 10 01	D. Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A. WORK UNIT
11. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
A. PRIMARY		6277IA		3E16277IA804		00	
B. CONTRIBUTING						041	
C. X-REFERENCE		CARDS 114F					
12. TITLE (Precede with Security Classification Code) ^a							
(U) Behavioral Variables in Autonomic Function and Disease in Military Personnel							
13. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
013400 Psychology 012900 Physiology 016200 Stress Physiology 02500 Clinical Medicine							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. FUNDING AGENCY		17. PERFORMANCE METHOD	
76 07		CONT		DA		C. In-House	
18. CONTRACT/GRANT				19. RESOURCES ESTIMATE			
A. DATES/EFFECTIVE: N/A				B. PRECEDING			
C. NUMBER ^a				FISCAL YEAR			
D. TYPE:				78			
E. KIND OF AWARD:				3			
F. CUM. AMT.				139			
G. CUM. AMT.				79			
H. CUM. AMT.				3			
I. CUM. AMT.				124			
20. RESPONSIBLE DOD ORGANIZATION				21. PERFORMING ORGANIZATION			
NAME ^a Walter Reed Army Institute of Research Washington, D.C. 20012				NAME ^a Walter Reed Army Institute of Research Div of Neuropsychiatry Washington, D.C. 20012			
ADDRESS ^a				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)			
RESPONSIBLE INDIVIDUAL				NAME ^a Hursh, CPT S.R.			
NAME: Rapmund, COL G.				TELEPHONE: (202) 576-2483			
TELEPHONE: (202) 576-3551				SOCIAL SECURITY ACCOUNT NUMBER:			
22. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Faden, MAJ A.I.			
				NAME: Cuthbert, CPT B.N.			
23. KEYWORDS (Precede EACH with Security Classification Code) ^a							
(U) Physiology; (U) Emotions; (U) Stress; (U) Autonomic Function; (U) Military Psychiatry; (U) Conditioning							
24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) This is a multidisciplinary effort addressing the development and use of laboratory models to define and describe the organ system responses and disease states caused by stressors in the military environment.							
24. (U) The techniques of operant and respondent conditioning will be employed in the production of models of both phasic and chronic psychological and emotional stress. Cardiovascular and gastrointestinal function will be monitored by electronic transducers and chronic indwelling catheters and fluid samples will be assessed for hematological and hormonal effects. Electrophysiological measurements of central and autonomic responsiveness will provide both a more accurate interpretation of similar data collected in studies with human volunteers and a source of hypotheses relevant to preventive and therapeutic intervention for cardiovascular and gastrointestinal disorders in military personnel.							
25. (U) 77 10 - 78 09 Studies of operant conditioning and stress with concomitant measurements of cardiovascular responses have been extended to blood flow and other cardiovascular indices with the baboon. Studies of operant-respondent conditioning have shown that history of experience with arousing stimuli influences current physiological and behavioral response to similar stimuli. Study of cardiac response during round-the-clock vigilance was initiated. During the fourth quarter a new investigator has renovated a laboratory and prepared research proposals for the study of gastrointestinal functioning during stressful behavioral tasks. Electrophysiological and anatomical studies of cardiovascular pathways in the spinal cord have been completed showing localization of an intraspinal sympathetic preganglionic pathway. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.							

DD FORM 1498
1 MAR 68

PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

1670

Project 3E162771A804 MILITARY PSYCHIATRY

**Work Unit 041 Behavioral variables in autonomic function and disease
in military personnel**

Investigators.

Principal: Hursh, CPT S.R.

Associate: Faden, MAJ A.I. and Cuthbert, CPT B.N.

Description.

Work within this unit has been directed toward the development of techniques of operant and respondent conditioning which can be employed as models of both phasic and chronic psychological and emotional stress and the application of the most current technologies for the monitoring of concomitant autonomic responses. The methodologies of both psychology and neurophysiology have been wedded in multi-level studies of behavioral, systemic, and neural changes which precede or accompany the development of psychosomatic disease. The first category of studies has focused on the control of autonomic responses by complex environment conditions requiring either intense and extended behavioral involvement or exposure to noxious and stressful conditions. Measures of cardiovascular or gastrointestinal function provide clues to the development of disease. The second category of studies has focused on the neural pathways and interrelationships which probably mediate the autonomic responses to these conditions.

Operant blood pressure and avoidance conditioning in the baboon: Work continued on a study comparing operant diastolic blood pressure conditioning and Sidman avoidance as methods for inducing and studying chronic arousal in the baboon. It was found that due to the difficulty of hand-shaping baboons with the physical configuration of the booth, direct avoidance training on one key was not feasible. Instead, the baboons were first trained on a lever for food reward, after which the food reward was alternated with periods in which presses on the lever turned off a loud, continuous Sonalert for 30 seconds. Each successive response postponed the re-introduction of the Sonalert for 30 seconds. Electric shocks to the tail were then programmed to occur every five seconds while the Sonalert was on; thus, the "Sonalert on" periods corresponded to the stimulus-stimulus (S-S) interval of a Sidman avoidance schedule, while "Sonalert off" periods were equivalent to the response-shock (R-S) interval. The final steps in this fading procedure were to discontinue Sonalert presentation altogether and to introduce masking noise as the discriminative stimulus for avoidance sessions. Food responses were then shifted from the lever to an adjacent round key, and made continuously available. Both baboons learned stable avoidance performance on this schedule. Shock was administered by two flat disk electrodes taped on the tail.

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Two baboons have been operated on to date. The first died several days following surgery. The second was run through the first phase of the experiment, the 72-hour avoidance session. Avoidance performance was maintained throughout the session, although rates of response began to drop after 24 hours and were at rather low levels by the end of the session. As expected, the baboon exhibited increased physiological arousal throughout the session. Although the data analysis is not yet complete, heart rate seems to have been particularly elevated; blood pressure appears to have been increased for the first several hours and then gradually returned toward baseline levels.

Apparently debilitated by the stress of avoidance, this animal also developed an infection and died about two weeks later. Efforts are under way to identify and remove the source of the bacterial contaminant, after which the third pilot baboon will be started on the experiment.

Behavioral and autonomic effect of an endogenous opiate: A single pilot monkey has completed a paradigm studying the behavioral and physiological effects of the peptide beta-endorphin. This hormone has attracted recent attention due to its action on the brain's opiate receptors, and may be an important component of the organism's stress response. Injections of beta-endorphin and control drugs were given 30 minutes into a 2.5-hour behavioral session in which the monkey worked for food on an alternating multiple fixed-interval, fixed-ratio schedule.

A 5 mg. dose of beta-endorphin resulted in a transient increase in blood pressure, followed by a decrease of about 15 mm which gradually recovered to stable, pre-drug levels over the next 20 minutes. A similar but more pronounced drop was observed following the 20 mg. dose; after the transient rise pressure sank from a baseline of 132/76 to a minimum of 105/55, and averaged 116/64 for the 10 minutes following the injection. Heart rate also showed a dose-response effect. The 5 mg. dose resulted in an immediate increase of about 32 beats per minute (bpm); this elevation was maintained for about 20 minutes, followed by a gradual return to baseline. Heart rate with the 20 mg. dose peaked at a level of 184 bpm, 40 beats above the pre-drug rate, and took about 10 minutes longer to return to baseline than with the lower dose. In contrast to the blood pressure and heart rate effects, respiration was virtually unaffected. No marked effects, particularly in a negative direction, could be seen following either dose. This negative result is encouraging in view of the respiratory depression induced by morphine and other alkaloid opiates.

The effects of beta-endorphin on fixed-ratio and fixed-interval performance were about the same, and somewhat shorter than expected. The maximum time of effect was estimated at five minutes for the 5 mg. dose and 7.5 minutes for the 20 mg. dose. The major effect on both schedules was a lengthening of the post-reinforcement pause rather than a decrease in response rate. No further effects were seen for the remainder of the session once responding recovered to normative values.

Next, two different doses of morphine were administered in order to compare the response to this synthetic opiate with that to beta-endorphin. The lower dose, .10 mg/kg, was apparently rather low in this preparation and had no observable effects on behavior or physiological measures. A dose of .57 mg/kg, however, was more effective. Behavioral responding following the injection was disrupted for the rest of the session on both the ratio and interval schedules, averaging only about 58% of pre-drug rates. Blood pressure decreased about 11 mm systolic and 7 mm diastolic, as with beta-endorphin; however, heart rate went down slightly rather than increasing. Respiration rate was also decreased for approximately 10 minutes following the morphine dose.

Finally, a .1 mg/kg injection of the alph-blocker phentolamine was given. As expected, this drug caused a physiological response similar to beta-endorphin, i.e., increases in heart rate and decreases in blood pressure. However, no effect on behavioral responding was observed; this suggests that the behavioral changes seen following beta-endorphin injections were not simply due to the animal's interoception of an altered physiological state, but rather to a presumably more direct, central effect.

Work during the next year with monkeys will replicate this study; in addition, a further study is planned using shock titration to assess the analgesic effects of beta-endorphin as compared to morphine.

Interactions between operant and respondent conditioning: This ongoing experiment was designed to study the effects of respondent conditioned stimuli superimposed upon operant baselines. Termination of this experiment has been delayed by the acquisition of a new computer system in the laboratory, which has necessitated suspension of physiological recording in the DRL experiment due to space limitations. Work completed before the computer's arrival tended to replicate and confirm earlier results. For one monkey working on a DRL schedule, a tone signaled the impending delivery of unavoidable shock. The physiological response during the conditioned stimulus was an increase in blood pressure and a large (40-60 beats per minute) decrease in blood pressure, the same pattern seen in earlier monkeys. Another monkey in which both food and shock reinforcement of two different CS's were initiated in the same session failed to show the marked heart rate deceleration, although blood pressure increased. These results suggest that the organism's physiological -- and, by inference, emotional -- response to unconditioned stimuli may be affected by the sequence in which they are introduced as well as by their affective quality.

An order difference is also suggested by an experiment in which a conditioned stimulus signaling free food is superimposed upon a Sidman avoidance baseline. Monkeys run this year have confirmed earlier results that classical conditioning of an appetitive CS begun during avoidance sessions results in an increase in response rate during the CS; when conditioning is first carried out at other times, however, a decrease in response rate results when the CS is finally introduced during avoidance.

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Three follow-up experiments are in progress. The first is a study with rats designed to test explicitly the order effect seen in the Sidman avoidance study. Currently, the rats have completed avoidance and food tray training and are entering the pre-conditioning phase of the study. A second experiment with monkeys, will be carried out to study further the unexpectedly large increases in blood pressure and heart rate seen in DRL monkeys throughout the session. Consideration of the DRL schedule has led to the hypothesis that a task requiring absolutely constant, unceasing attention is very stressful no matter what the reinforcer. An experiment will be performed to test this hypothesis by studying two levels of continuous attention during both negative and positive reinforcement; it is predicted that high attention demand will be more stressful overall than low attention demand, with negative reinforcement more stressful than a positively reinforced task. Stress will be operationally measured by recorded levels of physiological activity.

The third experiment involves an examination of circadian influences on DRL performance. Single and multiple daily sessions will be run at different times of day, and the behavioral and physiological changes recorded. As a comparison group, other monkeys will be run at identical times in a vigilance task. Initial shaping of subjects for both of these experiments has begun, and computer programs to run the experiments with the new computer system are in preparation.

Studies of gastrointestinal function and response to stress: This project, started late in FY78 upon the arrival of a new scientist, has seen significant progress in planning and preparation. A laboratory space has been prepared, test chambers have been modified, and essential measurement and control equipment have been purchased. Two principal studies are planned for the next year with the following objectives:

1. Determine if circadian/ultradian rhythms of gastric secretion and contractility can be documented.
2. Determine if feeding time alters these rhythms.
3. Determine if these rhythms can be modulated by stress.
4. Determine if secretagogues can generate chronic elevations of gastric secretion and what physiological factors protect against or contribute to the development of gastric ulcer.

Distribution of cardiovascular sites within the spinal cord: Physiologic studies were completed which examined the distribution of pressor, cardioacceleratory and inotropic sites within the cat spinal cord (1-4). These sites were found to be localized to the intermediolateral nucleus and highly lateralized to the right side of the spinal cord. This lateralization was strongly frequency dependent for blood pressure and cardiac contractility but not for heart rate.

Relationship of vertebral body to spinal segment: Physiologic studies which examine specific spinal segments require a knowledge of the relationship between spinal bony landmarks (e.g., spinous process, vertebral body) and spinal segments. Since such information had never

been determined for the cat, we undertook a gross anatomic study to determine these relationships.

Endotoxin shock: The potential role of endorphins in endotoxin shock was studied, using the specific opiate antagonist naloxone. Naloxone treatment was shown to reverse the hypotension induced by endotoxin and was able to block such hypotension when given prophylactically.

Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 041 Behavioral variables in autonomic function and disease
in military personnel

Literature Cited.

Publications:

1. Faden, A.I. and Woods, M. Lateralization of cardiovascular function within the spinal cord. *Neurology* 28: 332, 1978. (Abstract)
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3. Faden, A.I., Jacobs, T.P., Woods, M. and Tyner, C.F. Zona intermedia pressor sites in the cat spinal cord: right-left asymmetry. *Exp. Neurol.* In press.
4. Faden, A.I. and Petras, J.M. An intraspinal sympathetic preganglionic pathway: anatomic evidence in the dog. *Brain Res.*, 144: 358-362, 1978.
5. Faden, A.I., Woods, M. and Jacobs, T.P. An intraspinal sympathetic preganglionic pathway: physiologic evidence in the cat. *Brain Res.* In press.
6. Jacobs, T.P. and Faden, A.I. The relationship of spinal segment to vertebral level in the cat. *Anat. Rec.*, 190: 431, 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION		2. DATE OF SUMMARY		3. REPORT CONTROL SYMBOL	
				DAOC 6453		78 10 01		DD-DR&E(AR)336	
4. DATE PREV SUMMARY		5. KIND OF SUMMARY		6. SUMMARY SCTY		7. WORK SECURITY		8. RESEARCHING	
77 10 01		D. Change		U		U		NA	
9. NO. / CODES		10. PROGRAM ELEMENT		11. PROJECT NUMBER		12. TASK AREA NUMBER		13. WORK UNIT NUMBER	
A. PRIMARY		62771A		3E162771A804		00		042	
B. CONTRIBUTING									
C. CONTRIBUTING		Cards 114F							
14. TITLE (Precede with Security Classification Code)									
(U) Military Preventive Psychiatry									
15. SCIENTIFIC AND TECHNOLOGICAL AREAS									
003500 Clinical Medicine 013400 Psychology 021900 Physiology									
16. START DATE		17. ESTIMATED COMPLETION DATE		18. FUNDING AGENCY		19. PERFORMANCE METHOD			
76 07		CONT		DA		C. In-House			
20. CONTRACT/GRANT				21. RESOURCES ESTIMATE		22. PROFESSIONAL MAN YRS		23. FUNDS (\$ Thousands)	
A. DATES/EFFECTIVE: N/A				B. PREVIOUS		C. CURRENT		D. FUTURE	
E. NUMBER				F. FISCAL YEAR		G. YEAR		H. YEAR	
I. TYPE				J. AMOUNT		K. CUM. AMT.		L. YEAR	
M. KIND OF AWARD				N. YEAR		O. YEAR		P. YEAR	
24. RESPONSIBLE DOD ORGANIZATION				25. PERFORMING ORGANIZATION					
NAME: Walter Reed Army Institute of Research Washington, DC 20012				NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry Washington, DC 20012					
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Pursuit DR&E if U.S. Academic Institution)					
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26. GENERAL USE				27. ASSOCIATE INVESTIGATORS					
Foreign Intelligence Not Considered				NAME: Harris, LTC, J.					
				NAME: Schneider, CPT, R.					
28. REVISIONS (Precede with Security Classification Code)									
(U) Psychiatric Illness; (U) Military Adjustment; (U) Environmental Factors; (U) Social and Psychological Factors; (U) Stress									
29. TECHNICAL OBJECTIVE, 30. APPROACH, 31. PROGRESS (Pursuit individual paragraphs identified by number. Precede text of each with Security Classification Code.)									
<p>23. (U) This unit examines the dynamics of those specific factors within military organizations and environments that conduce to psychiatric illness, operate to produce psychiatric casualties and lead to the generation of dysfunctional behaviors and decrements in military performance. These studies have direct relevance for the development of programs of intervention and prevention and the development of effective techniques for the minimization of psychiatric casualties.</p> <p>24. (U) The methods of clinical psychiatry, social and clinical psychology, social anthropology and field epidemiology are used to identify factors that generate psychiatric casualties, behavior dysfunction and performance dysfunction and decrement in order to modify such factors or the relationship between them.</p> <p>25. (U) 77 10-78 09 Data gathered in studies of Artillery Fire Direction Centers are being analyzed to determine factors that mitigate or contribute to stresses of continuous performance. Field assays have been carried out with troops of the 82nd Airborne Division preliminary to projected studies of health factors involved in continuous performance and field deployment. Special emphasis is being accorded to the relationship of group structure to the stress response. Studies of fire fighters have been initiated in collaboration with the Department of Military Medical Psychophysiology, directed towards the development of models for chronic combat stress. Field studies of women in the Army and the socio-medical and medical psychological factors effecting both mental and physical health are in the process of being implemented. These studies will delineate factors of risk for female personnel. Studies of the relationship of group structure to physiology and the handling of stress in BCT-AIT and in deployed field units are under development and should commence in the coming fiscal year. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.</p>									

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PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

1077

Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 042 Military Preventive Psychiatry

Investigators.

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Description

Neuropsychiatric casualties have represented a major source of manpower loss in every armed conflict in which the United States Army has been involved. In times of peace the Army suffers significant personnel losses and costs as a function of behavioral dysfunctions, performance decrements, effectiveness deficits, psychosomatic illnesses, psychogenically based disorders and neuro-psychiatric diseases. Many of these losses and costs appear to involve predisposing risk factors that are parts of the general and human ecology of the Army. Unique aspects and demands of military life engender both strains and stresses that further the risk of the individual and the group for dysfunctional and ineffective behavior. The symptomatic and often costly responses to stressful events and factors in the military are in part determined by the health status and coping styles of the individual and in part by the social milieu in which stressful events are experienced. The interaction of the individual and group within this special set of ecological settings - ranging from the intense, life-threatening multiple stresses of combat to the daily stresses and strains of garrison and training - represent the central concern of this work unit. This unit examines the dynamics of those specific factors within military organizations and environments that conduce to psychiatric illness, operate to produce psychiatric casualties, and lead to ineffectiveness, the generation of dysfunctional behaviors, and decrements in military performance.

Central to this concern is the study of how the military social milieu organizes, shapes, reinforces and mitigates responses to stressful events. These studies have direct relevance for the development of programs of intervention and prevention and the development of effective techniques for the minimization of psychiatric and behavioral casualties.

1. Studies of Artillery Fire Direction Centers in Simulated Sustained Operations

Description

These studies were carried out jointly with the U.S. Army Research Institute of Environmental Medicine, Natick, Massachusetts and the Department of Military Medical Psychophysiology, WRAIR. The description of the studies was dealt with extensively in last year's Annual Report. Essentially four artillery fire direction centers from the 82nd Airborne Division carried out simulated sustained artillery operations under the observation of a number of investigators. Behavioral, physiological, and performance measures were taken.

Progress

During the past year, various analyses have been carried out and a number are still underway. These analyses are in general directed to the relationship between group structure and task loads in respect to performance as well as relationship of sleep patterns to overall performance. Data are currently being analyzed to deal with differences in communication patterns which emerged between periods of high and low task load. A repeated measures analysis of variance design is being used. In addition, complex demodulation analysis has been conducted of each individual's communication data. This technique provides a series of clock times during which changes in the normal rhythm of communications occurred and thus, better enables us to determine the impact on communication of task load. The archival video tapes from the two groups involved in an open ended simulation have been reviewed in order to evaluate and analyze the relationship between group behavior and structure, rhythmic patterns of communication, and task demand. The archival video tapes have also been examined to determine the total amount of sleep each individual got during the course of the simulation in order to help determine the relationship of sleep patterns to performance decrement and the decision of the group to withdraw from the simulation in terms of its perceived militarily ineffectiveness. The development of a series

of computer programs to effect complex demodulation analysis of communication data represented one of the major efforts in preparation for the analysis of the data acquired in the simulation. These programs may represent a major tool for the study of group performance in sustained operations modalities.

2. The Psycho-Social Aspects of Health and Illness of Women in the Army

Description

The purpose of this study, which is presently under development, is to describe the relationship between various psycho-social and organizational variables and the health status and effectiveness of performance and functioning of women in the Army. As of 1979, the Army plans to have approximately 50,400 enlisted women in its ranks, many serving in non-traditional MOSs. The impact of such increases in the number of female personnel on health resources, the possible consequences for individual and unit mental health and integrity are, at present, difficult to ascertain. Recent data on psychiatric hospitalization rates show that for 1971-73 the rate of episodes of psychiatric hospitalization for women was approximately twice that of males if drug and alcohol categories are excluded. Provisional analyses have shown that Army women had more days lost than men due to hospitalization and a greater non-effective rate in 1975 counting both physical and psychiatric hospitalizations. When gender specific illnesses were excluded, females continued to have a higher hospitalization rate than males. Similar results have been shown for the Navy. Past experience indicates that when mental health rates differentials exist for groups in the Army, major contributory factors are invariably to be found in organizational and unit forces and in psycho-social factors - particularly the stress provoking ones found in the military environment. The study under development projects extensive work in the field to: determine if such factors exist; isolate and describe them; and the development of preventive measures. The specific pilot research program designed to investigate in detail the health problems of female soldiers and how these compare with those of male soldiers and to produce a descriptive ethnographic study of women in the Army with specific focus on health has been developed. From the data collected in this preliminary investigation hypotheses will be developed for later definitive studies investigating gender differences in the health of soldiers. The research efforts will be concentrated on enlisted members stationed at Ft. Meade, Maryland. The

methodologies to be used include participant observation, in-depth questionnaires, health diaries and a study of outpatient records. Participants will be selected from the following units: Kimbrough Army Hospital to include the Community Mental Health Activity (CMHA), the Headquarters Command, the 76th Engineer Battalion, the 85th Medical Battalion, the 519th Military Police Battalion. Participant observers will be attached to the 519th Military Police Battalion and the Headquarters Command.

Progress

The following progress has been made: (a) the protocol has been approved; (b) the research technicians have undergone intensive training for the past six months; (c) the health diaries, the interview and demographic questionnaire as well as the health diary interview schedule are ready for testing; (d) unit commanders have been briefed with respect to the study; (e) initial data with respect to the mission and organizational structure of the participating unit have been obtained; (f) unit rosters are now being examined for the purpose of obtaining matched samples; (g) approval has been obtained from the Health Services Command allowing Kimbrough Army Hospital and the CMHA to participate in the study. Accordingly, (h) examination of medical records has begun.

Full scale participation of the other units awaits final U.S. Army Forces Command approval.

3. Studies of Small Groups in Chronically Stressful Occupations

Description

A collaborative study between the Department of Military Psychiatry and the Department of Military Medical Psychophysiology of Montgomery County Firefighters was launched during this fiscal year. This study directs itself to physiological and cognitive relationships of perception of stress and the stress response. For a full description, see the Annual Report of the Military Medical Psychophysiology.

4. Health Aspects of Deployment

Description

Military doctrine demands that Army units in garrison be able to deploy with optimal readiness and maximum strength in as

rapid a time as possible. Health problems, familial problems, and social and psychological problems presented as health problems have consistently been seen as factors mitigating against optimum troop deployability. During the course of the past fiscal year, information gathered by investigators of the Division of Neuropsychiatry has consistently demonstrated that these factors account for 10-15% of force strength which is not available for deployment to field exercises, REFORGER exercises, field testing, and other rapid transitions. Such factors are indicated by increases in sick call, requests for profile, requests to be removed from jump status in airborne units, and requests to be excused from participation in deployment exercises due to familial or economic problems. The majority of these requests are initiated through medical channels. Over the years a number of commentators in the social and behavioral sciences have postulated that the sick role, i.e., the validation of an individual as either ill or at physical or psychological risk for exacerbation of illness, represents the most socially acceptable avenue for withdrawal from or avoidance of situations perceived as stressful, potentially stressful or undesirable. The utilization of medical channels and the sick role by individuals to have themselves defined by both the military medical system and Command as non-deployable represents a continuous potential degradation of readiness. At present, a series of studies are in the planning and evaluation stage. These studies will be designed to come to grips with the effect of the above factors on deployment and deployability and the development of interventionary techniques.

Progress

During the past fiscal year, informal observation, information gathering, and discussions have been carried out primarily with personnel of the 82nd Airborne Division. It was discovered that between 10-15% of such personnel do not deploy with their units when they go to the field for either training or testing. Equivalent informal data has been gathered during the course of a study on jet lag relevant to units at Ft. Hood and Ft. Riley. Based on these preliminary observations, initial bibliographic work is now in progress for the development of specific studies. It is anticipated that such studies will concentrate on descriptions of the soldier's illness behavior, the conditions under which such behaviors are stressed, the function of such behavior for the soldier and the organization and the impact of such illness behavior on the military unit.

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The final design will undoubtedly include basic incidence and prevalence data and rates of change during periods of rapid deployment and transition. It will also include the study of classes of excuses used by individuals who attempt to define themselves as non-deployable - which may involve physical illness, psychogenic illness, psychosomatic complaints, emotional problems, family problems, etc. Such studies will also evaluate the outcome of illness behavior for the soldier, the role of the peer group in legitimating and supporting such behavior and the conditions under which such behavior is expressed in terms of organizational dynamics of the unit and the situational aspects of the individual soldier's life. It is anticipated that formal research protocols will be completed during the first quarter of the current fiscal year, and the research program initiated during the second quarter of FY79.

5. Attrition Studies

Description

The U.S. Army is presently suffering a significant problem of first-term attrition. Depending upon post, six to 12 percent of individuals entering BCT are discharged prior to the completion of their initial training. Over 40% of enlisted personnel are separated from the service prior to the completion of their first tour of duty. The attrition problem is a multi-faceted one involving medical psychological, medical, sociological, individual, organizational and stress response components. Present levels of attrition represent a chronic problem both in terms of financial costs, and maintenance of force levels and readiness of the Army. Initial studies are being formulated to look primarily at the relationship of stress and stress response to rates of attrition in basic combat training and advanced individual training. Members of the department have been briefed by personnel responsible for dealing with attrition at TRADOC and at Ft. Dix, New Jersey, one of the larger basic training centers in the eastern United States. It is anticipated that a series of studies involving medical, psycho-social, organizational and physiological parameters that may contribute to attrition as mediators of the stress response, will be undertaken during the course of the coming fiscal year. Such studies will be carried out jointly between the Department of Military Psychiatry, the Department of Medical Neurosciences, and the Department of Psychiatry, Uniformed Services University of Health Sciences.

Progress

During the last quarter of FY78, contact was made with the Commander at Ft. Dix, N.J. A series of informal observations and interviews are being carried out among cadre and trainees at that post. It is anticipated that pertinent variables for studies will be identified between the period Dec 78/Jan 79. Research protocols are targeted for completion by February 1979 and full field studies aimed at isolating factors involved in attrition and the identification of possible ameliorative techniques will commence by March of FY79.

6. Conditions of Psychiatric Casualties Under Catastrophic Conditions

Description

Military combat places the soldier in an environment which is considerably more stressful than any ordinarily experienced during time of peace. Thus in the absence of war the study of breakdown under extreme stress is made highly problematic. Among the few naturally occurring situations which may offer an opportunity to study psychiatric casualties under extreme stress is that of natural disasters. Natural disasters (such as hurricanes, floods, fires or tornadoes) place individuals in life threatening situations and may provide a civilian analogy to the stress of military combat.

Progress

Beginning in November 1977, a task force was formed for the purpose of reviewing the literature on civilian disasters and to evaluate the potential knowledge to be gained from field studies of disasters. Since formation of the task force, the literature on civilian disasters has been reviewed and several prominent authorities on disaster research, including Charles Fritz, Ralph Garrett and Russel Dynes, have been consulted concerning the feasibility and utility of such a project. Based on the literature review and consultations each task force member has produced written recommendations which are currently being synthesized into task force report. The final task force report is expected to be issued in the near future.

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Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 043 Military Stress: Health, Performance and Injury Factors

Investigators.

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Description

The soldier must perform in a multi-stress environment which affects health and performance. Research under this work unit is directed to a description of the stress factors, determination of the effects of these stressors on health and performance, and the design and implementation of counteractive measures to minimize these effects. Special attention is paid to stresses found in combat such as continuous performance, sleep deprivation, and temporal disorientation. The incidence of neuropsychiatric injuries and disease in a military population, the characteristics of these illnesses, counteractive measures to improve recovery, and the effect of residual deficits on performance are addressed in this unit. Performance is viewed as a continuum of human activity ranging from simple motor behavior to the most complex activity. A major attempt is made to distinguish between variations in performance which reflect; a) changes in an individual's basic sensitivity, efficiency, or capacity to perform a given task; and b) changes in the response biases, response criteria, or strategies employed in task performance. The research strategy utilizes field studies for the delineation of stress factors and their effects on health and performance, plus laboratory studies for technical development, to test experimental analogs, and to develop counteractive measures.

Progress

1. The Photometric Examination of Vascular Events

Optical techniques commonly used by psychophysicologists for the examination of vascular events were developed as alternatives to volumetric plethysmography. Since the optical signals appeared quite similar to those obtained by volumetric plethysmography, the optical techniques were referred to as photoplethysmography (the optical recording of changes in tissue volume).

The term photometry, may, however, be more appropriate for a technique which measures light rather than volume. In addition to being technically correct, photometry does not contain the physiological implications associated with the term photoplethysmography.

The relationship between photometric signals and vascular events must be defined in order to make inferences about vascular events from the photometric data. It has generally been assumed that the DC component of the photometric signal is an index of total blood volume, and the AC component is an index of pulsatile changes in blood volume. However, these assumptions have not been experimentally confirmed.

In an attempt to examine the physiological basis of the photometric signal, we have studied signals obtained with blood flow through an in vitro system. The general assumption that the AC photometric signal is an index of blood volume pulse may be tested by using a rigid glass cell in the system. If this assumption is valid, an AC photometric signal will not be observed in a rigid, non-distensible glass cell. We have shown that a pulsatile AC signal does occur in the rigid system when a pulsatile change in blood flow is produced by the pump. The sinusoidal photometric signal is similar to that obtained with the electromagnetic flowmeter. Therefore, the AC component of the photometric signal is not exclusively an index of blood volume pulse.

In order to determine the basis of the AC photometric signals obtained above, different fluids were examined using the in vitro system. When either hemolyzed blood or plasma is pulsed through the glass cell, there is no detectable pulsatile photometric signal. This suggests that the presence of red blood cells is necessary for the occurrence of a pulsatile photometric signal in a rigid system. Since the optical properties of blood have been shown to be dependent on blood flow, the AC photometric signal may be an index of pulsatile blood flow. From theoretical considerations of red blood cells as light scattering objects, the orientation of red blood cells with flow changes may account for the AC photometric signals observed.

Previous studies utilized rigid rather than compliant systems. The influence of vessel distensibility on photometric signals was examined with an agar-encased dog artery inserted in the in vitro system. Pulsatile signals resulted when whole blood was pulsed through the system. Pulsatile signals were also obtained when either hemolyzed blood or plasma was used. Therefore, these studies have demonstrated that both ordering

of red cells and vessel movement with flow contribute to the photometric signal.

These in vitro studies suggest that: a) pulsatile photometric signals are possible in a rigid system and are dependent on the presence of red blood cells; b) in a compliant system, vessel wall movement occurring with flow also contributes to the photometric signal; and c) the DC signal is directly related to increased flow, inversely related to vessel diameter, and is dependent on blood viscosity.

The conclusions derived from the in vitro system may assist in interpretation of photometric signals from human subjects. Blood flow in superficial arteries is predominantly pulsatile. Signals from superficial arteries are comparable to those obtained in the compliant system. Blood flow in the finger bed is a combination of pulsatile and steady flow with the pulsatile component predominating in the arterial circulation and the steady state component predominant in the venous circulation. The in vitro studies suggest that arterial events in the finger bed contribute to the AC component and venous events contribute to the DC component of the photometric signal.

2. Non-invasive Recording of Cardiovascular System Behavior

Field measurements of cardiovascular (C-V) function are sharply restricted by size, ruggedness, reliability, and noise artifact to the simplest and most time-proven techniques. Continuous recording of heart rate data (ECG) and intermittent sphygmomanometric blood pressure readings are the mainstay methods for discerning the C-V concomitants of environmental and behavioral events. However, neither measure has sufficient power to permit resolution of the dynamic mechanisms of any C-V system response. Additional measures of stroke volume, cardiac preload and contractility, peripheral resistance and blood flow distribution are desirable in order to circumscribe both C-V status and the nature of responses. Inferences as to neural and neurohumoral determinants of response likewise are dependent upon such a breadth of measurement. Since the latter determinants and the function of C-V effector organs (i.e. heart and vessels) have a well-established chronobiologic dimension, are potentially modulated by non-selective stressors, and in part determine the quality of response, e.g. physical and mental performance, effort has been invested in developing a technologic context within which field C-V data may be interpreted. Discussion of specific facets of an analytic "systems" approach to investigating the naturalistic (and clinical) interactions of the environment and the C-V system is in manuscript form (1).

In the laboratory, simultaneous recording via several non-invasive C-V recording techniques has been achieved for periods up to three hours on a beat-to-beat basis. ECG, phonocardiogram, apex cardiogram, carotid and radial artery tonometry, limb and thoracic impedance plethysmograph, digital infra-red photoplethysmography, and ultrasonic sphygmomanometry together have provided a number of continuously recorded indices of C-V system function. Quantitative changes and phasic interactions of these indices have been demonstrated in response to resting respiration and to such manipulation as Valsalva maneuver, mental arithmetic, cold pressor test, isotonic and isometric exercises, and postural change. Means of definitively recording, reducing, computing, and analyzing the resulting volume of data are in final stages of development, in the form of a digital recording system and a hard-wired device for computing significant parameters from pulse waveforms. Accumulation of data sufficient for response pattern analysis, detection of redundant indices, limits of error and other indices of data quality awaits final approval of an investigative protocol. Meanwhile, the recording/analytic system is finding application in describing data derived from pulsatile infrared photoplethysmographic measurements taken during on-going laboratory studies, described elsewhere in this report.

Investigation into the applicability, perhaps in the field, of other non-invasive sensors is underway. Among current possibilities are the new low-profile version of the infrared photoplethysmograph discussed in the prior section, a miniature, compliant monolithic tonometer being developed by Stanford Research Institute, and ultrasonic flowmeters. These and other techniques are discussed by members of this Department in Venables and Martin (2).

3. Blood Pressure Measurements on German Gun Crews During Fire Mission Exercises

In early 1978, we received an undocumented report from German colleagues of elevations, to abnormal ranges, of arterial blood pressure observed in 44 mm. bazooka gunners, following AM fire missions, and persisting for 2 - 3 hours. A literature review of "noise" vis a vis cardiovascular effects indicated: (1) a variable, probably non-specific, and highly transient cardio-deceleration in response to loud percussive noise, similar in pattern to the so-called alerting response; and (2) poorly controlled and anecdotal claims that noisy occupational environments pose a risk-factor for hypertension and atherosclerotic heart disease. Given this meager justification, and the opportunity had by Division members to observe, for other

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reasons, artillery field exercises, a series of heart rate and blood pressure measurements were made on members of DIVARTY, 82nd ABN, gun crews during the Spring of 1978.

In the first such "study", 23 members of a 105 mm howitzer battery had blood pressures recorded in the morning prior to deployment in ARTEP, and then 5-6 additional times during the field exercises, especially within minutes following fire missions. The rate of fire was quite low (< 10 rounds per day, per gun) interrupted by prolonged administrative lull periods. FDC team members were also measured. In all but a few instances, blood pressures observed were well within normal ranges; transient, mild (10-20 mm Hg) pressor responses were noted in a handful of instances, involving FDC members (100 meters away from guns) as well as gunners. A "clinical" impression was gained from these "casual" measurements that such elevations as were seen were coupled to a social-occupational context more than to gunfire events or noise, namely, instances of arguments, aim-errors, misfires, and undesired check-fire orders.

The second study, undertaken at Aberdeen Proving Grounds in May 1978, provided more formal data. Two gun crews of eight men each were required to "fanfire" 50-60 rounds from one of three guns. Over four successive days, each crew had a total of six missions, one on a 122 mm. howitzer, and 2 or 3 on 155 mm. and 105 mm. guns. Duration of each mission was 4-12 minutes (depending on the gun), and breech-measured overpressures ranged from 1.1 to 2.0 psi. Gun crews could be sharply divided into loaders (heavy work) and supervisors (light work) for each mission, although individual roles changed from gun to gun. On each of the four days, blood pressure and heart rate was recorded for crew members on the following schedule: (1) in barracks, 0600 ~ 0630 hrs; (2) in field prior to fire missions; (3) 5-15 minutes post-fire missions; (4) 30 min. post-fire missions; (5) 1-2½ hours post-fire missions; and (6) in barracks, 3-6 hours post-fire missions. Results for all 16 men, all missions are tabulated below:

	<u>0600</u>	<u>Group Mean Heart Rate (BPM)</u>				<u>3-6 Hrs Postfire</u>
		<u>Prefire</u>	<u>Postfire</u>	<u>30 mi Postfire</u>	<u>1-2½ hrs Postfire</u>	
<u>Heavy Workers</u>	72	69	97	83	82	76
<u>Light Workers</u>	72	70	81	83	77	76
<u>Group Mean Systolic Pressure (mmHG)</u>						
<u>Heavy Workers</u>	117	126	140	125	129	130
<u>Light Workers</u>	117	127	133	122	130	127
<u>Group Mean Diastolic Pressure (mmHG)</u>						
<u>Heavy Workers</u>	61	78	83	78	76	76
<u>Light Workers</u>	65	79	83	77	77	81

In summary, substantial elevations of heart rate and systolic blood pressure (less 50 with diastolic pressure) were observed, as expected, only in relation to physical work load, immediately after fire missions; elevations were greatest in the loaders, with heavier shell weight and longer mission duration (i.e., with the 155 mm. gun). No such elevations were sustained beyond 30 minutes post-mission, and by 1-2½ hours, blood pressure and heart rates were at or near AM (prefire) field measurements. No difference between responses to morning versus afternoon missions was discerned. Individuals with clinically borderline elevations of blood pressure (> 140/185) at rest were otherwise undistinguishable from those with lower resting pressures. Finally, sporadic elevations outside the general trends described were again accounted for by the social context of the given measurement.

These results coincide with observations last summer by Manning and Ingraham, of 155 mm. howitzer crews who fired up to 170 rounds over 11 hours of sustained fire. Thus, these attempts to confirm the German observations fail to suggest a clinically significant relationship between artillery fire and sustained modifications of cardiovascular responses. Such observational studies cannot adequately describe the physiologic consequences of the gunnery occupations, especially effects of combustion products, noise and blast overpressure, and significant health risks cannot be ruled out.

Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 043 Military Stress: Health, Performance and Injury Factors

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL	
				DA OC 6452	78 10 01	DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY ACTY	6. WORK SECURITY	7. REGRADING	8. DRG'S INSTR	9. SPECIFIC DATA - CONTRACTOR ACCESS	10. LEVEL OF SUM
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(U) Neuroendocrine Response to Military Stress							
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				NAME: Holaday JW			
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(U) stress (U) transmeridian desynchronization							
(U) neurotransmitters (U) hormones (U) peptides							
25. TECHNICAL OBJECTIVE, 26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To examine neuroendocrine and neurochemical correlates of stressors specific to the military environment. Types of stress to be studied will include extremes of heat and cold, psychological stress, as well as desynchronization of circadian rhythm.							
24. (U) Laboratory and field studies will examine the neuroendocrine response to psychological stressors (i.e., simulated combat exercises) ambient temperature extremes, transmeridian desynchronization, and continuous performance. These responses will be correlated with simultaneously-obtained data on performance decrement in the same subjects and work/rest schedules. Hormonal responses will provide bases for inferences concerning central nervous system neurotransmitter pathways essential to adaptation to stress, and optimization of work/rest schedules. This information is used to recommend pharmacologic and other therapies. Includes studies of physiological effects of hormones as well as assay development.							
25. (U) 77 10 - 78 09 Further studies with beta-endorphin (beta lipotrophic hormone 61-91 subunit) reveal it produces marked bradycardia and hypotension. Other studies show that naloxone temporarily reverses endotoxin-induced shock without affecting survival rates. We are succeeding in developing radioimmunoassays for several recently discovered endogenous peptides with potent physiological and potential psychological effects. Studies are in progress on the effect of contingency management on hormonal indices of stress and group behavior under continuous performance requirement. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.							

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Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

Investigators.

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I. Endocrine Profile as Indicator of Stress Response

Our approach emphasizes patterns of neuroendocrine response rather than the potentially misleading study of single neuroendocrine systems. A major immediate goal is to define in detail the characteristic hormone response profiles for various stressful stimuli: effect of multiple stressors, environmental factors such as continuous performance requirements, transmeridian desynchronization and/or ambient temperature, biological factors such as differences in sex or age, or effects of pain or neural injury. Understanding the impact of these factors often requires laboratory experiments which pursue leads developed in field studies and support work in the Departments of Military Psychiatry and Military Medical Psychophysiology. Hormonal response to stressors, for example, which can be observed in both field and laboratory studies, provides a critical link in facilitating coordination between those two efforts. Studies of neurochemical, neurophysiological and neuro-anatomical regulation of the body's response to stress provides a means of interpreting the field data. Rational design of prevention and treatment regimens for neuropsychiatric illness induced by military stress requires interpretation of the hormonal response profiles for stress in terms of the neurochemical systems producing the response. Hormone profiles are measured in clinical and animal studies as indicators of stress; they provide a basis for inferences about changes in brain function. For example, plasma prolactin levels provide a sensitive indication of the degree of central dopaminergic blockade induced by antipsychotic medication (1). Growth hormone secretion is stimulated by central catecholaminergic systems and is markedly increased during slow wave sleep (2,3,4). Thus, biochemical measurements may provide a useful measure of adequacy of rest periods during continuous performance studies. Additional neurochemical studies in animals will directly examine effects of stress in neurochemical activity of the brain and explore physiological, dietary, and pharmacologic methods for preventing stress-induced psychologic and physiologic disease.

A new and increasingly important area of research concerns the role of pituitary and brain peptide hormones in adaptation to environmental stressors. Most promising are peptide subunits of beta lipotropic hormone (BLPH). BLPH (MW 6900) was isolated from pituitary glands and characterized by Li in 1964 (5). Its major function was thought to be

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A. PRIMARY	62771A	3F162771A804	00	044			
B. CONTRIBUTING							
C. CONTRIBUTING	Cards 114F						
12. TITLE (Provide with Security Classification Code)							
(U) Neuroendocrine Response to Military Stress							
13. SCIENTIFIC AND TECHNOLOGICAL AREA							
012600 Pharmacology 002300 Biochemistry							
016200 Stress Physiology 003500 Clinical Medicine							
14. START DATE		15. ESTIMATED COMPLETION DATE		16. FUNDING AGENCY		17. PERFORMANCE METHOD	
76 - 07		CONT		DA		In-House	
18. CONTRACT/GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
A. DATE EFFECTIVE: N/A				PREVIOUS		B. FUNDS (in thousands)	
B. NUMBER				FISCAL		78	
C. TYPE				YEAR		3	
D. KIND OF AWARD				CURRENT		430	
E. CUM. AMT.				79		3	
21. RESPONSIBLE DOD ORGANIZATION				22. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research				NAME: Walter Reed Army Institute of Research			
ADDRESS: Washington, D.C. 20012				ADDRESS: Division of Neuropsychiatry			
				ADDRESS: Washington, D.C. 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Provide with Security Classification Code)			
NAME: Rapmund, COL G				NAME: Meyerhoff, JL, MD			
TELEPHONE: (202)576-3551				TELEPHONE: (202) 576-3559			
23. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Mougey EH			
				NAME: Holaday JW			
24. KEYWORDS (Provide with Security Classification Code) (U) stress (U) transmeridian desynchronization							
(U) neurotransmitters (U) hormones (U) peptides							
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Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

Investigators.

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Associate: G. Jean Kant, Ph.D., J.W. Holaday, Ph.D., MAJ
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d. NUMBER				78		3	
e. TYPE				79		3	
f. CUM. AMT.						430	
g. KIND OF AWARD						344	
23. RESPONSIBLE DOD ORGANIZATION				24. PERFORMING ORGANIZATION			
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* Available by contractors upon contractor's request.

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11. NO. / CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
A. PRIMARY	62771A	3E162771A804		00	044		
B. CONTRIBUTING							
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12. TITLE (Precede with Security Classification Code) ^a							
(U) Neuroendocrine Response to Military Stress							
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016200 Stress Physiology 003500 Clinical Medicine							
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76 - 07		CONT		DA		In-House	
18. CONTRACT/GRANT				19. RESOURCES ESTIMATE		20. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: N/A				PRECEDING		B. FUNDS (in thousands)	
B. NUMBER ^a				FISCAL YEAR		C. FUNDS (in thousands)	
C. TYPE				CURRENT		D. FUNDS (in thousands)	
A. KIND OF AWARD				78		3	
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23. GENERAL USE				ASSOCIATE INVESTIGATORS			
Foreign Intelligence Not Considered				NAME: Mougey EH			
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24. KEYWORDS (Precede EACH with Security Classification Code) (U) stress (U) transmeridian desynchronization							
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				DA OC 6452		78 10 01		DD-DNAE(AR)636	
4. DATE PREV. SUMMARY		5. KIND OF SUMMARY		6. SUMMARY SCTY ^a		7. WORK SECURITY ^a		8. REGRADING ^a	
77 10 01		D. Change		U		U		NA	
9. DESIG ^a		10. DOW'N INSTR ^a		11. SPECIFIC DATA CONTRACTOR ACCESS ^a		12. LEVEL OF SUM ^a		13. WORK UNIT	
		NA		<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO		A			
14. NO. CODES ^a		15. PROGRAM ELEMENT		16. PROJECT NUMBER		17. TASK AREA NUMBER		18. WORK UNIT NUMBER	
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B. NUMBER ^a				78		3		430	
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lipolysis and liberation of free non-esterified fatty acids. Recently, however, interest has focused on a BLPH peptide fragment composed of amino acids 31 through 91 of BLPH. This peptide has been named Beta-endorphin (β E). It is a very potent analgetic and hypothermic agent. Immunocytochemical studies have demonstrated intense staining for both α endorphin (α E) and β endorphin (β E) in the pars intermedia of the rat pituitary, with discrete cells staining positively in the anterior pituitary as well and with no staining in the pars nervosa (6). While the β endorphin antibody was highly specific for that peptide, the β endorphin antibody cross-reacted considerably with β lipotropic hormone (β LPH), which was previously demonstrated to be present in the pars intermedia and pars distalis (7). The function of the pars intermedia is not known with certainty. It has been associated with melanocyte stimulating hormone (MSH) (8), adrenocortical stimulating hormone (ACTH) (7) as well as β LPH (7). We are interested in assessing the role of the pituitary as the source of circulating endorphins and to ascertain the interrelationship among endorphins and hypothalamic, pituitary, and adrenal hormones. It is known that endorphins are found in the pituitary gland and in brain, they possess antinociceptive activity and we believe they may be involved in thermoregulation and cardiovascular shock as well. Several important questions remain to be addressed. Can endorphins be measured in blood? Are they released from the pituitary? If so, what regulates their release? What are the physiological and psychological roles of the endorphins, if any? The potential relevance of these questions to military medicine is illustrated by the data of Beecher (10) who studied the management of pain in soldiers wounded in combat in WWII. Of 215 severely wounded soldiers, 32.1% reported no pain and 25.6% only slight pain.

It has long been recognized that the cardiovascular system is exquisitely sensitive to the effects of exogenous opiates (11,12). In rats, less than one percent of the morphine dose necessary to produce antinociception results in significant hypotension and bradycardia (13). The endogenous opiate, Met-enkephalin, also has potent hypotensive effects when applied to the ventral surface of the brain stem in the cat (14). It is now known that another endogenous opiate, β Endorphin, is concomitantly stored and simultaneously regulated along with pituitary adrenocorticotropin (ACTH) (15,16) since stressors appear to result in the release of both peptides (15,16), it appears possible that stress-induced release of β Endorphin may result in alterations in blood pressure.

The administration of bacterial lipopolysaccharide endotoxin in animals has often been used as a model of human septic shock (17,18). It has been suggested that the profound hypotension induced by endotoxin is mediated through the release of endogenous substances (17,18). We hypothesized that endorphins may be one of the more important endogenous substances released by endotoxin. To test this hypothesis, in collaboration with the Physiology and Behavior Branch, we employed the specific opiate antagonist naloxone. Our results show that naloxone not only

rapidly reverses endotoxin-induced hypotension, but also prophylactically blocks its occurrence. Unfortunately acute injections of naloxone as well as infusions of 0.1 mg/kg, 1.0 mg/kg or 10 mg/kg failed to affect survival in rats subjected to endotoxin-induced shock.

The failure of naloxone treatment to improve survival in our studies of rat-endotoxin shock, despite its effect on blood pressure, suggested that factors other than cardiovascular may be critical determinants of survival in that model. Indeed, endotoxins are known to produce significant pathological changes in lung, gastrointestinal tract, and kidneys in addition to their effects on the cardiovascular system (17,18). It seemed possible that cardiovascular parameters might be more important determinants of survival in hypovolemic as compared to endotoxin shock and that naloxone might improve survival as well as blood pressure following hypovolemia. In a preliminary study, following exsanguination-induced hypovolemic hypotension, naloxone treatment rapidly improved mean arterial pressure and pulse pressure. These blood pressure changes were sustained and survival was significantly enhanced as compared to placebo treatment. The findings of this study may implicate endorphins in the pathophysiology of hypovolemic shock and suggest that narcotic antagonists may prove to be of therapeutic benefit in the treatment of shock.

In collaboration with the Physiology and Behavior Branch we have completed a preliminary study of the effects of intravenous injection of beta-endorphin on respiration, heart rate, blood pressure and operant performance in the Rhesus Monkey. Marked hypotension and tachycardia were observed and fixed-interval and fixed-ratio operant performance were briefly disrupted. A dose of phentolamine which produced similar hypotension and tachycardia failed to disrupt operant performance. Respiration rate and amplitude were unaffected by beta-endorphin in doses of 5 and 20 mg. Beta endorphin has been shown to have analgetic potency (19); we plan to compare the effect of analgetic doses of beta endorphin and morphine on respiration in monkeys. If βE produces less respiratory and circulatory depression than equianalgetic doses of morphine, βE might be preferable in some instances as a clinical analgetic. Studies have begun on the hormonal effects of injected beta endorphin. We are attempting to establish a reliable radioimmunoassay for plasma beta-endorphin and have succeeded in developing antibody activity.

One member of our group has completed an initial study of psychiatric symptoms during sustained operations during field maneuvers of the British Army. Nine percent of the sixty-six men participating reported visual distortions. These reports of visual distortions were obtained during interviews conducted throughout the course of the study. All these imperceptions occurred under conditions of severe sleep loss after three or more days of sustained operations. Differences were noted between the episodes occurring at night and those occurring during daylight. The night visions resemble the hallucinations reported in the studies of sensory deprivation and in anecdotal accounts

of prolonged isolation (20,21). One man who dreamed he was taking a reaction-time test was having a hypnagogic hallucination (22); another who thought that the sheep in a nearby field were polar bears was having a perceptual illusion (22). The night visions were the most common and the most disruptive of the hallucinations. Men saw night visions when it was totally dark and conversation was at a minimum, hence at a time when they were socially isolated and deprived of sensory stimulation. It seems clear that the night visions were more than benign epiphenomena of the sleep-deprived state, and that they, in and of themselves, confused the men and disrupted their performance. The dependence of the night visions on conditions of social isolation and sensory deprivation suggests that, if social isolation and sensory deprivation were reduced, performance during sustained operations would be improved. The hypnagogic hallucination and perceptual illusion occurred during the day, were brief, and did not disrupt performance. This suggests that they are indeed epiphenomena of the sleep deprived state and of no consequence to the conduct of sustained operations except as indicators of fatigue.

Collaborative studies with Dr. Joseph V. Brady at Johns Hopkins University have been examining the effect of contingency management on performance on a group task continuously performed over many days. This experiment studied the effects of two incentive conditions, *i.e.*, positive and negative reinforcement, on individual and social behavior within a three-person group residing in a programmed environment. Such effects were evaluated by comparisons between conditions of work-task productivity, ratings of interpersonal adjustment and of intrapersonal "mood" status, *e.g.*, depression, and other measures. Performance was monitored on the Alluisi Multiple Task Performance Battery within a duty station which could be occupied by subjects one at a time on a self-determined rotational basis continually over a 24 hour period. Heart rate, skin potential, frontalis EMG, and skin temperature were recorded during work, and total 24 hour urine volume was collected for cortisol analysis. The substitution of aversive for appetitive control produced lowered productivity, episodes of disruptive behavior, reports of depression and anger at experimenters but no changes in urinary free cortisol in preliminary experiments. Mean daily urinary free cortisol levels did correlate positively however, with mean daily scores on the multiple task performance battery (five concurrent tasks). Studies are planned on the effect on performance and hormone excretion of the addition or deletion of a group member in mid-experiment. Studies will be done on female as well as male groups and may later include groups containing both male and female subjects.

A study of basic training at Fort Dix has been initiated in collaboration with the Departments of Psychiatry, Division of Neuropsychiatry and the Uniformed Services University of the Health Sciences. Preliminary observations have begun preparatory to planning a study which will test the usefulness of urinary hormone assays in predicting success or failure of recruits in coping with stress in basic training.

Additional studies have been planned with the Department of Medical Psychophysiology on the hormonal indices of transmeridian desynchronization and effects of prophylactic measures.

Collaborative studies with Dr. Joseph Y. Bousquet, Jr. at the University of California, San Diego, have been completed. The purpose of this study was to determine the effect of a 12-hour shift in the timing of sleep on the circadian rhythm of body temperature. The study was conducted in a controlled environment where the subjects were kept on a constant schedule of sleep and wakefulness. The results showed that the circadian rhythm of body temperature was shifted in phase with the shift in sleep timing. This finding is consistent with the hypothesis that the circadian rhythm is a fundamental biological process that can be influenced by external factors such as sleep and wakefulness.

A study of the effects of a 12-hour shift in the timing of sleep on the circadian rhythm of body temperature was also conducted. The study was conducted in a controlled environment where the subjects were kept on a constant schedule of sleep and wakefulness. The results showed that the circadian rhythm of body temperature was shifted in phase with the shift in sleep timing. This finding is consistent with the hypothesis that the circadian rhythm is a fundamental biological process that can be influenced by external factors such as sleep and wakefulness.

Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

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Project 3E762771A804 MILITARY PSYCHIATRY

Work Unit 044 Neuroendocrine Response to Military Stress

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)636	
3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8A. ORG'S INSTN ^a	8B. SPECIFIC DATA- CONTRACTOR ACCESS ^a	8C. LEVEL OF SUB A. WORK UNIT
77 10 01	H. Term	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	
10. NO./CODES ^a	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER			
A. PRIMARY	62771A	3E162771A304	00	045			
B. CONTRIBUTING							
C. CONTRIBUTING	Cards 114F						
11. TITLE (Precede with Security Classification Code) ^a							
(U) Follow-up Studies of Human Volunteers who received Psychoactive Substances							
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a							
003500 Clinical Medicine 013400 Psychology							
13. START DATE		14. ESTIMATED COMPLETION DATE		15. FUNDING AGENCY		16. PERFORMANCE METHOD	
76 07		78 09		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		19. PROFESSIONAL MAN YRS	
A. DATE/EFFECTIVE: N/A				B. PRECEDENCE		C. FUNDS (in thousands)	
B. NUMBER: 0				FISCAL YEAR		77	
C. TYPE: 0				CURRENCY		8	
D. KIND OF AWARD: 0				E. CUM. AMT.		464	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
NAME: Walter Reed Army Institute of Research Washington, DC 20012				NAME: Walter Reed Army Institute of Research Division of Neuropsychiatry ADDRESS: Washington, DC 20012			
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish NAME if U.S. Academic Institution)			
NAME: Rapmund, COL G.				NAME: Marlowe, D.H., Ph.D.			
TELEPHONE: (202) 576-3551				TELEPHONE: (301) 427-5210			
21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER:			
Foreign Intelligence Not Considered				ASSOCIATE INVESTIGATORS			
				NAME: Garcia, LTC J.			
				NAME: Redmond, MAJ D.			
22. REVISIONS (Precede each with Security Classification Code)							
(U) Lysergic Acid Diethylamine; (U) Epidemiology; (U) Clinical Sequelae							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
<p>23. (U) In the period of 1956-1969 some 600 military volunteers received LSD in the course of experiments with chemical warfare agents carried out at Edgewood Arsenal AMC. The purpose of this work unit, mandated by the Surgeon General, has been a follow-up medical evaluation to determine whether there have been any clinical or other sequelae to the ingestion of this agent.</p> <p>24. (U) A medical and epidemiological follow-up examination and study has been designed utilizing the various disciplines of clinical medicine, psychiatry, psychology and epidemiology.</p> <p>25. (U) 77 10-78 09 At the direction of OTSG the materials for medical evaluation and the final examination module for individuals who received psycho-active substances has been turned over to the OTSG and HSC for completion of the examinations. All data files have been transferred to the responsible officer from the OTSG and responsibility for examinations of all remaining former subjects as well as the analysis of data have been transferred to the OTSG representative. The design and piloting phase of this follow-up being completed, the obligations and participation of WRAIR have been terminated as of this past fiscal year. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.</p>							

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1784

Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 045 Follow Up Studies of Human Volunteers Who Received Psychoactive Substances

Investigators.

Principal: David H. Marlowe, Ph.D.

Associate: LTC Juan M. Garcia, MC, MAJ Steven D. Gilbert, MC, MAJ David A. McFarling, MC, MAJ Daniel Redmond, MC, CPT George D. Bishop, MSC, Glenn T. Gurley, BA, Stanley Hall, BA, Frederick W. Hegge, Ph.D., Joseph M. Rothberg, Ph.D., Helen Sing, MS, SP5 Marie A. McCarty, SP5 JoAnne Smith.

Description

During the period 1956-1969 some 600 military volunteers received Lysergic Acid Diethylamide (LSD) in the course of experiments carried out at Edgewood Arsenal, AMC. In 1975 OTSG mandated that the WRAIR Division of Neuropsychiatry take responsibility for the development of a follow up medical evaluation of these individuals. The Division of Neuropsychiatry was also charged with assessing whether or not a scientific study was feasible that would determine if there were specific clinical sequelae causally related to the ingestion of LSD under the auspices of researchers in chemical warfare.

Progress

WRAIR and the Department of Military Psychiatry participation in Follow Up Studies of Human Volunteers was terminated during the course of the first half of FY78. Examination modules developed at WRAIR, data files, medical files, and all other pertinent information were turned over to OTSG, USA. OTSG set up a special office to carry out the post-pilot medical examinations of the remaining former volunteers. This work unit has been terminated as a WRAIR activity.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY					1. AGENCY ACCESSION ^a		2. DATE OF SUMMARY ^a		REPORT CONTROL SYMBOL DD-DR&E(AR)636		
3. DATE PREV SUMMARY		4. KIND OF SUMMARY		5. SUMMARY TYPE		6. WORK SECURITY		7. REGRADING ^a		8. ORIGIN INSTR ^a	
77 10 01		D. Change		U		U		NA		NL	
9. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER		10. WORK UNIT NUMBER			
A. PRIMARY		62771A		3E162771A804		00		046			
B. CONTRIBUTING											
C. XEROX/OTHER		CARDS 114F									
11. TITLE (Precede with Security Classification Code) ^a											
(U) Medical Factors Limiting Soldier Effectiveness											
12. SCIENTIFIC AND TECHNOLOGICAL AREAS ^a											
016200 Stress Physiology 013400 Psychology											
13. START DATE			14. ESTIMATED COMPLETION DATE			15. FUNDING AGENCY			16. PERFORMANCE METHOD		
77 10			Cont			DA			C. In-house		
17. CONTRACT/GRANT						18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS		B. FUNDS (in thousands)	
A. DATES/EFFECTIVE: N/A						PRECEDING					
B. NUMBER ^a						FISCAL YEAR		78		60	
C. TYPE						CURRENT		79		60	
D. KIND OF AWARD						E. CUM. AMT.					
19. RESPONSIBLE DOD ORGANIZATION						20. PERFORMING ORGANIZATION					
NAME ^a Walter Reed Army Institute of Research Washington, D.C. 20012						NAME ^a Walter Reed Army Institute of Research US Army Medical Research Unit-Europe					
ADDRESS ^a						ADDRESS ^a HQ, USAMEDCOMEUR APO NY 09102					
RESPONSIBLE INDIVIDUAL						PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)					
NAME: Rapmund, COL G.						NAME ^a Ingraham, MAJ L.					
TELEPHONE: (202) 576-3551						TELEPHONE: (AVN 435) 740					
21. GENERAL USE						SOCIAL SECURITY ACCOUNT NUMBER					
						ASSOCIATE INVESTIGATOR					
						NAME: Manning, MAJ F.					
						NAME					
22. KEYWORDS (Precede EACH with Security Classification Code)											
(U) Epidemiology; (U) Stress; (U) Psychiatry; (U) Human Volunteer; (U) Soldier Effectiveness											
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)											
<p>23. (U) To identify factors in the military organizational, social, psychological and physical environment that create risk for and conduce to psychiatric breakdown, behavioral dysfunction, psychosomatic and physical illness as they impact on individual and unit effectiveness and consume health care resources.</p> <p>24. (U) The methods of epidemiology, including records analysis, population and demographic analysis, questionnaire and field and cohort studies as well as observational methods are employed to develop requisite data.</p> <p>25. (U) 77 10-78 09 A significant portion of the previous FY was devoted to establishing the performing organization's European unit, negotiating office space and filling it with personnel, equipment, and supplies as well as discovering and exploring likely contacts and areas for formal investigations. One such exploration involved screening records of all personnel departing USAREUR via administrative discharge during April 77. Non-structured interviews were also conducted. Observations are presently underway in a FA battalion, in response to a request from V Corps Artillery for advice on performance during continuous operations. Also underway is a post hoc study of the personalities of drug overdose victims and their unit environments. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.</p>											

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1 MAR 66

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1706

Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 046 Medical Factors Limiting Soldier Effectiveness

Investigators

Principal: MAJ Larry H. Ingraham, Ph.D., MSC

Associate: MAJ Frederick J. Manning, Ph.D., MSC

DESCRIPTION.

Work within the unit during the first year of its existence explored the fertility of this field setting for inquiries into environments (social, organizational, psychological and physical) that create risk for psychiatric breakdown, behavioral disfunction, and illness, and especially their impact on individual and unit effectiveness and health care resources. These exploratory efforts were aimed at securing a broad knowledge of the European environment that will be used to plan more definitive studies. This work was accomplished by targeting on militarily significant problems identified by commanders as pressing concerns within the European theater of operations. Three such concerns guided our work.

BIOMEDICAL ASPECTS OF CONTINUOUS OPERATIONS.

The ability of soldiers to perform around the clock in a sustained engagement is a matter of serious concern to military commanders planning for a major military operation in Europe. V Corps Artillery became concerned about their ability to sustain high rates of fire called for in planning scenarios, and requested assistance in evaluating the abilities of their personnel to meet the projected rates of fire demands. We responded with an extensive interview and observation schedule to provide us a detailed background from which we might rank by order of importance those factors most likely to degrade performance. Observations were made both in garrison and in the field and included both active duty service members and their dependents in a single field artillery (cannon) battalion. Questionnaire data was collected and personnel and medical records were screened to provide data for correlations with critical units events like the Annual General Inspection, ARTEPs, ammunition up-loads, and participation in REFORGER exercises. We submitted an interim report to the Corps in July 1978; detailed analysis of the observations and preparation of the final report is currently underway.

EPIDEMIOLOGY OF ADMINISTRATIVE DISCHARGES

Personnel who leave the command prior to their expiration of their normal tours of duty for administrative reasons impose a serious

drain on the available manpower within the command. Nearly all of these discharges stem from some form of maladaptive behavior. For example, one quarter to one third of these individuals are discharged for reasons of inappropriate drug or alcohol use. These individuals, along with an unknown number of others who are processed through mental hygiene clinics pose a continuing demand for health care resources. Responsible preventive psychiatry is currently hindered by the little epidemiologically significant data available on this population to include such basic information as a detailed demographic profile or the distribution of military units generating those discharges. Therefore, we screened the personnel records of all individuals leaving the command for administrative reasons during one entire month. In addition, we conducted in-depth group and individual interviews with a 10% sample of the population that focused on military careers from motivations toward entering the Army, through basic and advanced training, experiences in Europe, and perceptions upon leaving the service. These data are currently being analyzed.

PREVENTION OF DRUG OVERDOSE CASUALTIES.

Soldiers who use illicit drugs continue to receive much attention from the European Command. One of the most powerful indicants of increasing illicit drug use is increasing numbers of drug-related deaths. As deaths increase, there is an increasing demand for medical services to manage the near-death overdose casualties. Little information was available of epidemiological significance regarding drug overdose casualties. Therefore, we are conducting a study of drug overdose casualties using interview techniques. The method used is the psychological autopsy, originally developed to assist medical examiners in specifying cause of death. It involves interviewing the friends, leaders, and associates of the casualty with the objective of reconstructing insofar as possible the events and circumstances immediately preceding the overdose. The objectives of the study are to provide psychological profiles of the overdosed, and to seek ways of preventing recurrence. Only casualties who are admitted to the hospital in seriously ill or very seriously ill status have been considered during the initial stages of inquiry. Data collection and analysis is ongoing and will continue through FY 1980.

Project 3E162661A804 MILITARY PSYCHIATRY

Work Unit 046 Medical Factors Limiting Soldiers Effectiveness

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION		2. DATE OF SUMMARY		REPORT CONTROL SYMBOL	
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3. DATE PREV SUMMARY		4. KIND OF SUMMARY		5. SUMMARY SCTY		6. WORK SECURITY		7. REGRADING	
77 10 01		D. Change		U		U		NA	
8. NO. / CODES		9. PROGRAM ELEMENT		10. PROJECT NUMBER		11. TASK AREA NUMBER		12. WORK UNIT NUMBER	
A. PRIMARY		627110		3E 16 2771A804		00		047	
B. CONTRIBUTING									
C. CONTRIBUTING		Cards 114F							
13. TITLE (Precede with Security Classification Code)									
(U) Military Psychiatry Epidemiology									
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003500 Clinical Medicine 013400 Psychology									
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76 07			CONT			DA		C. In-House	
19. CONTRACT GRANT				20. RESOURCES ESTIMATE				21. PROFESSIONAL MAN YRS	
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B. NUMBER				FISCAL YEAR				78	
C. TYPE				CURRENT				6.5	
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								288	
22. RESPONSIBLE DOD ORGANIZATION					23. PERFORMING ORGANIZATION				
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24. GENERAL USE					SOCIAL SECURITY ACCOUNT NUMBER				
Foreign Intelligence Not Considered					ASSOCIATE INVESTIGATORS				
					NAME: Dattel, W.E., Ph.D.				
					NAME: Rothberg, J.M., Ph.D.				
25. KEYWORDS (Precede each with Security Classification Code)									
(U) Military Adjustment; (U) Psychiatric Illness;									
(U) Epidemiology; (U) Behavioral Dysfunction; (U) Psycho-Social Factors									
26. TECHNICAL OBJECTIVE (26. APPROACH, 27. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)									
23. (U) This unit examines military organizational, social, psychological, and environmental factors that create risk for and conduce to psychiatric disease, psychosomatic illness, behavioral dysfunction and physical illness as they effect Army personnel and impact of care giving agencies.									
24. (U) The methods of epidemiology, including records surveillance, population and demographic analysis, questionnaire and field and cohort studies as well as methods of the psychological and social sciences are used to delineate environments of risk for psychiatric illness and periods of special risk for such illness at critical points in the career of the soldier.									
25. (U) 77 10-78 09 Analysis of the past and present patterns of psychiatric disease, illnesses with a psychosomatic component and behavioral dysfunctions is underway utilizing materials available through IPDS and other DA reporting systems. Studies are being carried out analyzing the psychological and health problems of women in the Army. Studies are being carried out on the epidemiology of neurological syndromes following penetrating wounds. Studies have been carried out reviewing aspects of therapeutic psychotropic drug use during the Viet Nam conflict. Analysis of cohorts representing selected accessions of personnel during F*72 to determine precursors of dysfunctional behavior continues. Data gathering for the development of an instrument to study the relationship between social supports and health has been completed, analysis and development will continue during the coming year. Planning and negotiations for the study of the relationship of stress to attrition in BCT and AIT have been completed and studies will proceed during the coming fiscal year. Studies of socio-medical and medical-psychological consequences of deployment are in the process of development. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sept 78.									

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14710

Project 3E162771A804 MILITARY PSYCHIATRY

Work Unit 047 Military Psychiatric Epidemiology

Investigators.

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Description

The military environment places markedly different demands and strains upon its population than do civilian environments. These demands and differences in terms of individual and unit effectiveness and performance, mental and physical health, and behavioral disruption and dysfunction have chronic effects in peacetime. In periods of deployment and combat, such stresses may have acute affects on the capability of units and individuals to perform their missions. This unit examines military organizational, social psychological, and environmental factors that both create risk for and militate against psychiatric disease, psychosomatic and physical illness, behavioral dysfunction and disruption of performance as they affect Army personnel and impact on care giving agencies. The methods of epidemiology, including records surveillance, population and demographic cohort studies and methods of the psychological and social sciences are used to delineate factors conducing to risk as well as mitigation for such illnesses, disruptions and dysfunctions.

1. Battle Injuries and Associated Neurologic Disorders Sustained by Active Duty Army Personnel During the Vietnam Conflict

Description

A descriptive analysis of battle injuries sustained in previous conflicts may be of value in predicting the effects of combat on health and recovery patterns and long term impact on the Army Medical system. A research proposal has been implemented to perform an epidemiologic study of battle injuries and associated neurologic disorders sustained by active duty Army personnel during the Vietnam conflict.

Progress

Data records from the Individual Medical Records (IMR) and Individual Patient Data System (IPDS) of hospitalizations due to Battle Injuries (BI) were provided by the Patient Administration System and Biostatistical Activity (PAS&BA) of the Patient Administration Division of the Health Services Command. These data records were processed by WRAIR and selected portions of the records were transmitted to the Veterans Administration (VA) Beneficiary Identification and Location System (BIRLS). The information returned from BIRLS was processed at WRAIR and selected portions were transmitted back to PAS&BA to be processed with the IPDS. This later processing provided data records of hospitalizations (1971 through 1977) of individuals whose Social Security Account Number (SSAN) is either a) the same as an IMR or IPDS BI with that SSAN, or b) the same as the SSAN of a BIRLS record selected because the first or second Service Number (SVN) is the same as the SVN of an IMR BI.

There were 126,067 distinct SVNs which were extracted from the PAS&BA BI data records and sent to the VA BIRLS. The BIRLS located 82% (103,742) of those records in their files. All of the matches contained a name but only 45% (57,223) also contained a SSAN. The SSAN from the BIRLS as well as those previously available from PAS&BA BI records were processed to give 95,843 unique SSAN which were transmitted to PAS&BA to be matched against the IPDS.

There remain 68,844 BI SVN which do not have matching SSAN. Microfilm copies of the Army payroll records which contain both SSAN and SVN are available at the Fort Benjamin Harrison Finance Center.

The internal validity of the individual data columns of the IMR have been tabulated (256 possible codes for each of the 80 positions in each of the 354,289 records). The eight column

field for the body of the SVN had non-numeric characters for 86 IMR records. These non-numeric characters prevent linkage of these records to any others. The remaining 354,203 IMR records had data columns that ranged from 0% to 27% missing and/or invalid values.

The internal consistency of the IMR records has been partially tested by use of the data columns which indicate the total number of data cards for the current medical event and the sequential position of the current card within that total number. Review of several hundred data records suggests that the inconsistencies can be adequately resolved. This analysis is continuing.

Evaluation of the external consistency of the IMR against the BIRLS uses the correspondence (or lack thereof) of similar data appearing in both files. Provisional analysis comparing age and date of birth, dates of service with date of admission and with date of death show 13% inconsistent records.

External validity is being evaluated through the use of battle injury reports that are independent of the IMR and IPDS systems. Such reports have been located in the Vietnam Studies and in medical and scientific journal articles. The popular press is currently being reviewed for comparable reports.

2. Psychiatric Hospitalization of Females in the Army

Description

This is a four year prospective study which will determine the extent to which psychiatric hospitalization and diagnosis differ between male and female soldiers during the course of their first term enlistment. A further comparison will be made of hospitalization outcome of soldiers receiving traditional basic training versus soldiers receiving sex-integrated basic training. As this study progresses it is anticipated that it will generate hypotheses which can be tested in epidemiological field studies.

Progress

Each of the seven basic training posts within CONUS, as a result of a dictate from HQ TRADOC, submitted rosters of all male and female enlistees entering the Army in the month of June 1977.

In addition, rosters of all male and female soldiers who participated in the Basic Initial Entry Training Test (BIET) at Ft. McClellan, Alabama completed in October, 1976 and male and female basic trainees undergoing traditional training during the same period were submitted and received.

Cohort definition is not yet complete. Machine readable lists have been produced of soldiers participating in traditional and gender integrated basic training at Ft. Jackson in October 1976 and of soldiers drawn from seven reception stations in June 1977. Initial attempts to identify these 25,793 soldiers within the MILPERCEN system have located 78%. Attempts to identify the sources of the mismatches are continuing.

The formats for the anticipated reports have been developed and computer programs have been written to produce the reports. Cross-tables of the demographic characteristics recorded in the Enlisted Master File have been generated from the located records of soldiers who entered the Army during the cohort definition period.

3. The Expectation Fraction Instrument

Description

Research carried out in the psycho-social aspects of psychiatric epidemiology during recent years has indicated that availability and quality of an individual's social support network - serves a major function determining the health outcome for that individual in the sequence of responses to both acute and chronic stress. Much of the literature of military psychiatry indicates, as well, that the face to face social supports provided by the soldier's primary group serve in a powerful manner to affect his ability to successfully cope with the multiple stress of deployment and the battlefield. The Expectation Fraction Instrument represents an initial attempt to develop a quantifiable instrument for assaying and inventorying the self-perceived quality and frequency of contact of an individual with his network of "significant others" - those who comprise his social support system. The successful development of such a model would enable us to relate critical psycho-social processes and the individual's perceived relationship to his social support system, to protection and susceptibility to breakdown while under conditions of stress as well as to the evolution of health outcomes.

Progress

An initial version of the Expectation Fraction Instrument was developed. A pilot study to test its reliability and construct validity was completed this summer. An initial sample of 50 to determine test-retest and internal reliability was completed and analyzed. A more extensive sample of 100 to determine the instrument's validity was collected and is now in the process of being analyzed. The subjects for both samples were drawn randomly from the civilian and military staff of WRAIR. Initial analysis established that external reliability product-moment correlations are above +0.80. The internal Kuder-Richardson reliability correlations are also above +0.80. The external and internal reliability of the EFI is therefore apparently high. Initial analysis also indicates that a step-wise linear gradient appears to exist between individual perceptions of social support and responses made on the General Well Being and Hopelessness Scales. More exacting validity measurements and further analysis are currently being conducted.

4. Career Outcome Study

Description

Urine positivity for drugs of abuse is assumed to be a risk factor for psychiatric, and physical illnesses as well as behavioral dysfunctions and ineffective performance. This study was designed to assess the long range behavioral implications of urine positivity and its potency as a factor of selection for risk for health and behavioral problems and to evaluate the medical evaluation procedure following designation of an individual as urine positive. A cohort demonstrating this presumed risk factor and controls who entered the Army during 1972-73 was defined in order to prospectively study their individual medical and military careers. The cohorts consist of 1967 individuals with positive urines and 2432 negative urine controls. The rate of matching was slightly over 80% for each cohort.

Progress

Analysis by the members of this cohort for specific precursors of illness and dysfunctional behavior is continuing. There are no special results to be reported for this year.

5. Psychotropic Prescription Medication in Vietnam

Description

In 1967, a questionnaire was mailed to Army physicians performing out-patient services for troops on duty in Vietnam. This questionnaire presented a list of 28 psycho-active medications and requested the physician-respondent to indicate all uses of the drug listed. Of 233 questionnaires mailed to non-psychiatrists, 110 were returned. 6 of 21 psychiatrists responded as did two Navy psychiatrists. This questionnaire represented the only attempt at sustained data-gathering on the impact of the therapeutic use of psycho-pharmacologic agents in the combat environment. These materials have been analyzed in collaboration with the Office of the Consultant in Psychiatry.

Progress

The principal value of the data is to be found in the nature of the sample of patients for whom the drugs were prescribed. We know of no other study published in the open literature of psychotropic drug prescriptions on military troops serving in a combat zone.

The shortcomings in the study are many. The physician response rate was poor, particularly among the psychiatrists. The data that were reported were not always exact nor meticulously recorded, seeming at times to be drawn from memory rather than medical records. Number of patients served and number of prescriptions written were necessarily confounded in the analysis.

Keeping in mind the nature of the sample and the limitations of the study, the major findings of the study were:

(1) The best estimate for the annual psychotropic drug prescription rate was 12.5 per cent of all prescriptions.

(2) Psychiatrists prescribed more psychotropic drugs per prescribing physician than did the primary care physicians, with such a by-discipline prescription ratio being 1.5 (i.e., 20.2 to 13.7).

(3) Gastroenteritis was the most frequently occurring condition to be treated with the psychotropic drug armamentarium of the primary care physician. Compazine was the drug of

choice for this condition and the results of the treatment were judged by the treaters to be very good.

(4) Excluding gastroenteritis, the most frequently occurring condition treated with psychotropic drugs by either primary care physicians or by psychiatrist was anxiety. It was treated principally with minor tranquilizers and the results noted were quite good.

(5) Combat fatigue, a subset of the anxiety cases, was treated most frequently with a major tranquilizer and the results were usually good.

(6) A wide variety of conditions were treated with the psychotropic agents, especially by the primary care physicians. In general the results described were quite efficacious, with possible exceptions in the case of anorexia and headache.

(7) Primary care physicians tended to view the results obtained from the medications used more favorably than did psychiatrists.

(8) Psychiatrists tended to prescribe the psychotropic drugs in heavier doses and for longer periods of time than did the primary care physicians.

(9) Disallowing the highly frequent use of Compazine for gastroenteritis, one of out every five psychotropic drugs prescribed by either the primary care physicians or the psychiatrists was a sedative/hypnotic. Obviously the newly established generation of tranquilizing medications has not completely replaced the barbiturates, at least not in 1967.

(10) Presenting conditions for which psychotropic drugs were prescribed were rarely labeled as depression by the primary care physicians, and seldom labeled as such by the psychiatrists.

PUBLICATIONS

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Rothberg, J.M., & Datel, W.E., "Military Outcome of Trainees: a Partial Replication". Military Medicine, 143:111, 1978.

Rothberg, J.M., and Chloupek, Robert J., "A Longitudinal Study of Military Performance Subsequent to Civilian Drug Use". American Journal of Public Health 68: 743-747 (1978).

Project 3E162771A805

MICROWAVE INJURY

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION ^a	2. DATE OF SUMMARY ^a	REPORT CONTROL SYMBOL DD-DR&E(AR)838	
3. DATE PREV SUMMARY ^a	4. KIND OF SUMMARY ^a	5. SUMMARY SCTY ^a	6. WORK SECURITY ^a	7. REGRADING ^a	8. DISTR INSTR ^a	9. SPECIFIC DATA CONTRACTOR ACCESS ^a	10. LEVEL OF SUM ^a
77 10 01	D Change	U	U	NA	NL	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO	A WORK UNIT
10. NO./CODES ^a		PROGRAM ELEMENT		PROJECT NUMBER		TASK AREA NUMBER	
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B. CONTRIBUTING						041	
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11. TITLE (Precede with Security Classification Code) ^a							
Biological Interactions with and Hazards of Microwave Radiation							
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014100 Radiobiol 012900 Physiol 014000 Rad Chem 017000 Wave Prop 013400 Psychology							
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71 07		CONT		DA		C. In-House	
17. CONTRACT/GRANT				18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	
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D. KIND OF AWARD ^a						874	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION			
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21. GENERAL USE				SOCIAL SECURITY ACCOUNT NUMBER			
				ASSOCIATE INVESTIGATORS			
				NAME ^a Jacobi, J.H.			
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22. KEYWORDS (Precede EACH with Security Classification Code) ^a							
(U) Microwave Hazards; (U) Biophysics; (U) Dosimetry; (U) Bioeffects; (U) Military Medicine; (U) Psychology							
23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)							
23. (U) To provide technical and medical information to the Surgeon General, system developers and agencies responsible for safety standards in order to protect the health and effectiveness of military units and affected civilian populations in microwave and RF environments. This requires analysis of the biophysics and bioeffects attributable to non-ionizing radiation under laboratory conditions which reasonably simulate operational exposures.							
24. (U) To perform basic and applied research on the problem of microwave and RF interactions with biosystems at all levels of analysis from the cellular and molecular to metazoan physiology, pathophysiology and behavior. This requires development of measurement systems for dosimetric analysis ex vacuo, in vitro and in situ; the evaluation of frequency, power level, polarization and modulation as important parameters of the radiation; and the use of low level energy to assess the functional state of cells and tissues.							
25. (U) 77 10 - 78 09 Progress has included the demonstration of feasibility for non-invasive microwave dosimetry using scattering parameters and time delay spectrometry; development of network analysis methods for high speed, broad band measurement of permittivity in biological tissues; development of methods for improved spatial resolution in microwave images of modeled biological targets; preliminary studies to assess functional states of cells and molecules in vitro by electromagnetic analysis; development of microwave transparent electrodes for temperature measurement and induced electric field strength in situ; and development of high power pulse exposure facility for radar simulation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.							

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1719

Project 3E162771A805 MICROWAVE INJURY

Work Unit 041 Biological effects and hazards of microwave radiation

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I. Introduction

The Department of Army is a major consumer and developer of communications and electronic systems which employ a large segment of the electromagnetic spectrum. The frequencies actually employed extend from the audio band (in the order of 10^3 Hz) to millimeter wave bands (in the order of 10^{11-12} Hz). In terms of total pieces of deployed equipments, the numerically most prominent band is the HF band (10^6 to 10^7 Hz) where nearly one-quarter of a million sources exist (1). These items of equipments are primarily tactical communications devices and are typically of relatively low radiated power (in the order of 10^{-1} W). The most prominent high power band (in the order of 10^3 to 10^5 W) is the radar L band (in the order of 10^5 Hz) where approximately 200 sources exist in CONUS alone. A typical application for these sources is deployment as air defense radars, i.e. target acquisition and tracking radars. The band of most active development is in the millimeter wave bands (in the order of 10^{10} to 10^{12} Hz) (2). Typical uses for these sources are as terrain mapping and guidance radars. Total radiated power is most often moderate, in the order of 10^1 to 10^2 W. However, high power sources are becoming available such as the recent development of a 105 KW, CW gyrotron operating at 28 GHz.

Biological effects are definitely known to extend from 34 MHz to 100 GHz. The lower frequency extreme (34×10^6 Hz) represents the frequency of optimal energy transfer to a grounded 1.7 M man with long axis parallel to the electric field. The upper frequency extreme (ca. 10^{11} Hz) represents the frequency of coherent thermoelastic resonances. There are a myriad of factors that come into play at these and intermediate frequencies. Prominent among these is depth of penetration, coupling efficiency and focusing factors in both the transmitted field and the geometry of target organs. It is likely that frequencies beyond the two extremes described above will prove to be important, especially in scientific application.

The Surgeon General of the United States Army has direct responsibility under AR 70-1 for the final determination of operator and personnel safety in the presence of these emitters. The Surgeon General's resources for this task are chiefly USAEHA and USAMRDC. The enterprise is greatly complicated by the fact that no international agreement exists on the standards of safe exposure. This lack of agreement is the result of

many factors, not all of them scientific. There are, however, numerous discrepancies of fact, not to mention interpretation, that exist within Western scientific circles which are sufficient to engage our attention. Prominent among these are the role of frequency dependent bioeffects, the role of modulation parameters, and the role of combined stressors in the form of multiple simultaneous emitters as electronic warfare continues to grow faster than any other area of material development. Thus, the role of EMR in the modern battlefield represents a prodigious problem for which a paucity of objective, relevant scientific information exists to assist a rational management of the Army Medical Department's responsibilities.

A major fraction of the task resides within the Medical R&D Command since even if detailed descriptions of all emitters under all circumstances of use did exist, we would still be unable to defend by objective means a medical evaluation of the risk and hazards presented by all or even a large portion of the conditions likely to be present on the modern battlefield. This already difficult situation is further complicated by the fact that adversary forces employ emitters which are obviously not under our control, and that the CE/EW environment must be considered as an element in the event that adversary forces deploy CBW or directed energy weapons.

Finally, there is the potential that diagnostic, therapeutic and scientific applications exist for the use of RF and microwave energy. This possibility does not contradict the role of hazards research. Past experience offers many examples where beneficial properties of agents were exploited only after they were first identified by their hazards. This includes a number of "poisons" also used in chemotherapies; and, of course, x-ray which has proven carcinogenic properties. Indeed, the history of medicine often suggests that one could infer useful actions only after biologic activity was confirmed by toxicity.

The subject which this department is charged to address is, therefore, one of bewildering complexity. We must consider a vast range of frequencies, power levels, modulation parameters and multiple sites of action involving all organ systems. The pressing question is how to begin.

We do have considerable experience to guide a research plan. The Tri-Services EMR project spanned a period of nearly 3 decades. Yet the lack of reproducible results have limited any generality that this period of activity could bring to bear on today's problems. Part of the explanation for this shortage is the lack of adequate instrumentation for quantification of absorbed dose. As a result, this department has many major program elements directed toward dosimetry. These include programs for development of implantable, microwave transparent electrodes for temperature and induced electric field measurement, calorimetry, and dosimetric analysis by radiofrequency tomography as detailed in the project summaries to follow. A corollary to this aspect of the research

program is its contrast to earlier thought where internal field measurements were regarded as "senseless".

"Standing wave patterns in the body make it senseless to talk about energy flux in the body and to use it as a measure of dosage. All dose or dose rate statements must refer to field or flux values in the distant field defined sufficiently far from the body to be affected by its presence (3)."

Another element is our continued interest in measurement of the electromagnetic constitutive parameters of tissues under physiological conditions. It is abundantly clear that permittivity measurements in autopsy material at room temperature neglect target organ geometry, physiological state, and any functional responses to the radiation (of special importance in this regard is the role of local vasodilation in response to local hyperthermia). Many elements of department program are directed to this problem. Prominent among these are the in situ permittivity, cellular and molecular programs (cf. program summaries). It is important to realize that this represents a significant departure from prior thought typified by the conclusion published in the 1970 Symposium Proceedings on the Biological Effects & Health Implications of Microwave Radiation from which I quote: "no further work is needed in regard to absorption coefficients (4)."

The department program strongly supports the view that it is important to acquire an understanding of basic mechanisms by which EMR interacts with biosystems at the cellular and molecular level. We believe that this approach is fundamental if we are ever to acquire predictive value. Given the rate of development of Army systems which use EMR and the complexity of the situation described in earlier paragraphs, a research approach directed solely by the appearance of new systems in the equipment inventory is doomed to failure. It is imperative to acquire sufficient understanding of the basic mechanisms that we can contribute to EMR parameter selection at the time of development rather than recommend remedial measures after vast sums of money are already committed to a deployed system.

The final point of general scientific strategy is the importance of frequency and modulation parameters. The recent modification of our high power transmitter will give us the first opportunity to study high power pulsed fields which reasonably simulate those produced by a radar system. The importance of frequency is illustrated in a number of studies where frequency is optimized for the system under examination. This includes the taste aversion studies, the cell membrane studies and the whole of the permittivity studies where we are³ extending our range of network analysis capabilities to extend from 10^3 Hz to 18×10^3 Hz. Again it is important to realize that this is a major departure from prior thought whereby the exposure frequency was most often 2450 MHz simply because tubes at this frequency are less costly due to their application in industrial heating.

II. Administrative Activities

A. Patents

This report period represents a high water mark of patent activity. In the present reporting period, 6 patent disclosures were filed with the Chief, Patent Prosecution Branch, DA Intellectual Property Department. Of these, 5 have already been filed as patent applications, and of these, claims already have been officially approved by the U.S. Patent Office for 2 patents relating to electrode development and microwave imagery in biosystems. We expect that patents will actually be issued on these two applications (#842,138 and #842,137) sometime in CY 79. Further, we expect successful action on all those patent items so far disclosed. In light of all the other competing demands on our productivity, a few words may be in order to explain why we elected to further dissipate investigator effort on patent applications. We cannot deny or dispute the fact that these applications represent a significant segment of the total effort of the department leadership (ca. 180 hours thus far). Neither can we dispute the cost to DA for patent prosecution (ca. \$7-9K per application), nor the uncertainties regarding approval of claims. However, these applications do represent major developments in an area of emerging technology which will prove to be financially valuable in orders of magnitude above the cost of patent prosecution. Consider for example, the area of biomedical microwave imagery. The volume of business in x-ray imagery for biomedical application has been estimated at 5-10 billions of dollars per year in the world market. Since patents are a major mechanism for sole source procurement, if DA supports the R&D to produce patentable inventions and fails to secure patent rights, then contractors may effectively twice and thrice charge DA for technology which by merit it already owns. Further justification for this effort is the establishment of a historical record of research products which often more fully represents the author's/inventor's aspirations/accomplishments than is the case with referenced journals.

B. Construction

This has been a year of major administrative accomplishments. Primal among these are activities which relate to two major construction programs. One was remodeling of the exposure facilities and animal quartering at Bldg 502/503 in the Forest Glen Annex. This year has seen the continuation of a 14 month, \$1M building project to install environmental chambers to expand and improve animal quartering facilities, to install environmental control for the 3 anechoic exposure chambers of recent vintage, to construct an exposure facility for the high gain elliptical antenna and to demolish our earliest "anechoic" chamber (the first installed in the USA for biological analysis of microwave exposure).

Concurrently, plans have been developed and implemented to add a vertical feed to Chamber C with highly accurate circular polarization. This modification will allow animal exposure without the confinement/

restraint presently necessary to assure a stable dose. Similarly, the Cober transmitter is being modified to operate as an L band radar simulator. Since the vast majority of high power Army emitters operate in the pulse mode within the L band, we have planned for a 1.2 to 1.5 Megawatt pulsed klystron to operate at 1.3 GHz with pulse widths from 1 to 20 μ sec (in two steps) at duty cycles up to 0.01 for average powers in the range of 10 to 15 KW.

The importance of these facilities improvements can hardly be overstated. In the case of the pulsed transmitter, this is the first time in the over 12 year history of this department that any free field exposure facility has been available that approximates modern radar emitter conditions which may be uniquely hazardous to biosystems. Likewise, the previously poor environmental control greatly frustrated and in some cases prevented biological studies in free field circumstances.

Secondly, we are preparing for another ca. 1 year, ca. \$700K remodeling in the reactor room of Bldg 40 to divide the present space into 2 floors. This will replace our present small and temporary lab/office space with larger, permanent and more usable facilities for those aspects of the program which do not require free field exposures. These program elements include DART, MIC electrode development, as well as cellular and molecular studies which take place in vitro.

These facilities enlargements will permit full development of the extramural program by dedicating the 502/503 locale to free field exposures and relieve the competitive inhibition between intramural and extramural programs by transferring a major fraction of the former to Bldg 40 where support services and collaborators are more readily available. We are also in the process of developing a system for assessing charges to extramural users for facilities utilization, professional services rendered, as well as marginal expenses.

In addition to the disorder in facilities, this was a year of personnel disruption. The two phenomena are related to the extent that one staff position has been devoted effectively full time for the last 14 months to the reconstruction of Bldg 502/503. In addition, the remaining department leadership has devoted significant blocks of time and effort to the Bldg 502/503 project as well as the Bldg 40 project. This condition was aggravated by the fact that still another investigator was absent on months of TDY for training and research which were basically unrelated to the microwave mission. Lastly, we have yet to bring the department up to authorized strength due to recruitment delays for one professional civilian slot and one military technician slot.

C. Tri-Services Electromagnetic Research Program (TERP)

Army contributed to a revised version of TERP programmatic document. Army refused to sign the prior agreement on the basis of certain fundamental

disagreements. The prior document was roundly criticized. It no longer served as a framework for additional agreements and a completely new document was drafted in a series of meetings at NAMRI and WRAIR culminating in final meeting at USAFSAM prior to a Joint Medical Research Council briefing in October 1978.

Among the plans made at TERP meetings were those for a series of TERP sponsored meetings on topics considered of pressing importance. The first of these is to be a symposium on the blood brain barrier as a microwave target organ. This is to occur in the fall of 1978 and to be sponsored by the Navy. The second symposium is to take place in the middle trimester of 1979 on the subject of mechanism of microwave interaction with biosystems. This symposium will be under Air Force sponsorship. The following year (1980), Army is to host a symposium on microwave imagery as applied to complex dielectrics, especially biosystems.

D. Office of Telecommunication Policy (OTP)

The Executive Office of the President contained an EMR information clearing house and policy coordination organization known as the Office of Telecommunications Policy. Under the aegis of the Office of Science and Technology Policy (also of EOP), OTP was reorganized within the Department of Commerce as the National Telecommunications Information Agency (NTIA). NTIA continues its role of interagency coordination. In this vein, Army completed its reporting requirements to NTIA in this period.

E. EPA Intercession

In the spring of 1978, EPA sent letters to all major agencies of the federal government concerning, among other things, the impact of standards setting activities on Army materiel development, Army occupational standards, and Army operations. This request for information was routed through the TERP mechanism for action. The Surgeon General of the Army determined that it was not within the purview of USAMRDC to answer these questions. These questions were routed through Deputy Chief of Staff for Research, Development and Acquisition (DCSRDA), Deputy Chief of Staff for Operations (DCSOPS), and Health Services Command (HSC) for opinions concerning impact on materiel development, operations, and occupational settings, respectively. USAMRDC retained specific responsibility for biomedical research relating to standard establishment (cf. a more complete MFR on the subject for details). The EPA plans to reach some decision concerning the need for a statutory microwave exposure standard and to recommend some power density value (likely to be 1 mW/cm^2 , an order of magnitude lower than the present advisory ANSI C.95 standard).

Army participated in a general planning session called by EPA and it prepared written responses to the EPA questionnaire insofar as USAMRDC could so do with SGO guidance. Other aspects were referred to subordinate

commands of DARCOM, FORCECOM, and TRADOC.

It is apparent that OSHA also plans to act in this area. In fact, jurisdictional disputes are likely to be legion since Commerce is also developing a posture for standards setting in this area. Thus, not only EPA and BRH are active in this area, but also OSHA, NIOSH, NBS and NTIA.

F. Congressional Appearances

The United States Senate continued its high level of interest in the Radiation Oversight Hearings which began in the summer of 1977. This interest took the form of 3 written inquiries following the summer hearings. In addition, during this report period, a special hearing was requested by Senator Cannon and other members of the Senate Armed Services Committee as well as the Senate Science Subcommittee which was to be conducted with Top Secret classification. The objective was to explore questions remaining in the minds of staff and Senators concerning classified aspects of State Department and Army activities in EMR.

In the case of the State Department, these questions centered on events surrounding the alleged bombardment of the American Embassy in Moscow.

In the case of the Army, the questions pertained to classified research surrounding project PANDORA, the destruction of some laboratory notebooks, the transfer of classified research reports, and certain differences of opinion concerning interpretation of experimental findings (cf. a classified MFR for further details). We were able to provide the Committee with a complete inventory of the documents that were destroyed in 1973/74, copies of the secret research reports, copies of proceedings of several PANDORA meetings concerning interpretation of experimental results. A lively discussion ensued which culminated in the promise for further written questions and a general reproachment toward objectivity concerning the referenced documents.

III. Research Activities: Intramural Program

A. Cellular and Molecular Applications of Permittivity Analysis (L.E. Larsen, J.H. Jacobi)

The HF band permittivity analysis system with its application to cellular physiology and induced pathophysiology as described in the 1977 Annual Report has resulted in a publication which was included in a special issue of IEEE Microwave Theory and Techniques on medical applications of electromagnetic radiation. This work is summarized as an abstract below:

"A technique for the electromagnetic analysis of physiological and pathophysiological states in cell suspensions is presented. The technique is based upon high speed automatic network analysis in the HF band for

measurement of complex permittivity. The results demonstrate that changes in HF band permittivity dispersion may be related to physiological and drug induced pathophysiological states of the cell membrane. Mechanical disruption of the cell membrane by sonication obliterates the HF band dispersion of permittivity that is present in undisrupted cells. The effect of species, suspending medium and temperature were systematically analyzed in erythrocyte suspensions in order to aid comparison between published studies of red cell preparations."

This system was also applied to a study of bases, base pairing, nucleotides, and nucleic acids as described in the 1977 Annual Report. This work was presented to the 1978 annual meeting of the Biophysical Society. A publication is in preparation, but delays have been experienced because one member of the research team has accepted another position outside of WRAIR.

B. Measurement of Complex Permittivity of Biological Materials (J.H. Jacobi)

A paper describing the HF permittivity analysis system has been accepted for publication by IEEE Transactions on Instrumentation and Measurement. This work is summarized as an abstract below:

"A method of measuring the real and imaginary parts of the complex permittivity of materials (particularly liquids) is presented. The method includes a new calibration procedure that uses a material with known permittivity to calibrate the system. Data is presented from 110 MHz to 500 MHz for water, ethyl alcohol, and ethylene glycol. Considerations of the size of the capacitor with relation to a wavelength are discussed."

This year the technique of automatic network analysis (ANA) was applied to frequency bands both above and below the HF band. The bands below 1 MHz extending down to audio frequencies were the subject of a feasibility study for ANA application to 4 terminal measurements. Four terminal measurements are necessary at frequencies below ca. 1 MHz due to serious artifact from interfacial polarization when electrolytes are present in the sample. A study of existing methods disclosed serious technical shortages which when taken in perspective of available intramural resources lead to a decision to transfer this portion of the project to the FY 79/80 extramural program, leaving only the ANA application to the intramural program.

The extension to frequencies above the HF band are centering on the range between 2 and 4 GHz. Several prototype sample chambers were built and evaluated. These began with an extension of the lumped element technique (used successfully in the HF band) which proved to be completely unsatisfactory in the S band (indications in the literature notwithstanding). This was followed by a series of chambers based on "infinite" loss transmission lines. This technique proved to be better

than the former method, but it was still unusable in its early forms due to higher order modes produced by the high k loading presented by high water content biological specimens. Later versions appear to be distinct improvements over prior types, but full evaluation is not yet completed due to a shortage of coaxial line construction materials which do not present significant permeability. We expect to solve the materials problem and complete evaluation of the technique early in CY 79. Details of the procedures and applications are proprietary and the subject of DA patent proceedings.

C. Dosimetric Analysis by Radiofrequency Tomography (DART)

1. Scattering Parameter Imagery of Biological Dielectrics (L.E. Larsen, J.H. Jacobi)

This project has as its ultimate objective the noninvasive, three dimensional mapping of microwave energy absorption as a function of spatial variations in the complex permittivity of biosystems. These spatial variations are detected by their effect on the magnitude and phase of the scattering parameters S_{12} and S_{22} (complex reflection and complex transmission coefficients) as functions of space for various targets. The present report period has seen major accomplishments in this project. These consisted of the construction, installation and digital control of an extremely precise electromechanical scanner for closed loop positioning of water coupled S band antennas. In addition, several prototype antennas were designed and constructed to further explore means for resolution improvement and to study techniques for element stacking preliminary to development of a water coupled phased array. This included the development of miniaturized, water coupled elements with rectangular and circular apertures as well as the double ridged version.

Furthermore, scattering parameter imagery was collected for both canine and porcine renal specimens which were scanned as isolated organs. Although our analysis is incomplete due to our limited available digital image display and digital image analysis capabilities, a half tone display developed by Jacobi and low line density isometric displays do confirm that interspecies differences in renal geometry are preserved; and, more importantly, it appears that the method can distinguish the renal cortex and renal pelvis as well as part of the collecting system. The disclosure of the details of procedure and application must await completion of pending DA patent action.

Lastly, program plans were formulated and a contractor was selected via the RFQ mechanism for development of a water coupled phased array to begin late in this report period. The chief advantage of this system is that it will reduce data collection time for a 64 by 64 image from ca. 300 min. to ca. 2 min. Further increase in scan speeds will be possible with improved switching methods which may be retrofitted to the "existing" system to permit scan times of ca. 0.1-0.2 minute. In this way,

multifrequency analysis becomes possible prior to specimen degradation.

A paper which described the early work with dielectrically loaded antennas has been accepted for publication in Medical Physics. This paper is summarized as an abstract below:

"A system for the generation of microwave images from homogeneous and heterogeneous dielectric targets is presented. The argument is posited that microwave interrogation may address uniquely relevant features of biological targets. Dielectrically loaded antennas, electro-mechanical scanning and the methods of microwave network analysis were employed."

Also, a paper describing the water coupled antenna (double ridged, rectangular aperture) has been accepted by IEEE Transactions on Microwave Theory and Techniques. This paper is summarized as an abstract below:

"This paper describes a method of significantly improving the resolution of systems used for interrogating the spatial variation of permittivity of biosystems at S-Band. The basic principle employed is to contract the wavelength of the interrogating radiation and reduce the physical aperture of the interrogating probes by immersing the transmitting antenna, receiving antenna, and the target into a material with a high dielectric constant; namely, water. The antenna design is described and line scans employing transmitted and reflected energy are presented."

2. Microwave Time Delay Spectrometer Image of Biological Dielectrics (J.H. Jacobi, L.E. Larsen)

The time delay spectrometer was also subject to considerable developmental effort to improve spatial resolution in studies with phantom tissue dielectric targets. As a review of first principles, this is a method of characterizing biosystem targets by measurement of propagation delay as function of space. The time delay is estimated by measurement of instantaneous frequency differences between two chirped signals, one of which propagates through the target and the other through a reference path. The details of procedure and application may not be disclosed pending DA patent completion, but suffice it to say that Microwave Time Delay Spectrographic (MTDS) imagery was collected on targets which were totally uniform (i.e. isodense) to x-ray. The spatial resolution with the water coupled S band antenna appears to be consistent with that previously determined for scattering parameters imagery; that is ca. 5 mm. Time resolution remains at ca. 40 picoseconds of differential delay.

Futhermore, MTDS imagery has been collected for isolated organs. This data with fresh renal specimens of both canine and porcine origin disclosed the ability to image the difference between renal cortex and

renal pelvis. In addition, there are indications that portions of calyces and collecting system may be visualized.

A paper which described the early work with dielectrically loaded has have been accepted for publication in Medical Physics. This paper is summarized as an abstract below:

"A method is described which overcomes the problems of multipath propagation and range ambiguity that is suffered by the single frequency continuous wave microwave imaging system described in part I. This technique is essentially a variation of chirp radar techniques which have been adapted to time delay and attenuation measurements through a target. The feasibility of discriminating between paths whose differential time delay is on the order of 100 picoseconds is demonstrated. Further, the need for small physical aperture in the transmitting and receiving antennas is demonstrated."

Also, a paper describing the water coupled antenna (double ridged, rectangular aperture) has been accepted by IEEE Transactions on Microwave Theory and Techniques. This paper is summarized as an abstract below:

"This paper describes a method of significantly improving the resolution of systems used for interrogating the spatial variation of permittivity of biosystems at S-Band. The basic principle employed is to contract the wavelength of the interrogating radiation and reduce the physical aperture of the interrogating probes by immersing the transmitting antenna, receiving antenna, and the target into a material with a high dielectric constant, namely water. The antenna design is described and line scans employing transmitted and reflected energy are presented."

3. Image Display (J.H. Jacobi, M.E.T. Swinnen)

A further area of effort in the DART program has been the development of image display hardware and software for the microwave imagery. These consist of four systems, only one of which is complete at the present time. The only fully functional system is one based on a bistable or half-tone display using the Hewlett Packard 5451A. Coarse isometric plots are also available with this system. The system next to reach completion is a high line density isometric display which offers high speed data transmission from the processor/controller to achieve real time display, real time rotation of perspective, depth cueing and hardware magnification/minification. This system has been built with a parts cost of ca. \$7K, and it offers most of the salient features of similar commercial systems costing ca. \$40-60K. The next two display systems were the subject of an RFQ for development of a digital grey-scale display and a grey-scale hardcopy with a number of innovative features.

D. Red Blood Cell Permeability Changes (P.V. Brown, L.E. Larsen)

Eastern block reports continue to appear which purport to demonstrate that red blood cells have membrane permeability changes consequent to microwave energy exposure (5). The basic mechanisms involved are also relevant to the question of possible blood brain barrier alterations. The average level of energy needed is below the U.S. safety standard. Several Western investigators have tried to replicate the results, without success (6). It was felt that exposure conditions and methods were not comparable since dosimetry could not be determined. In addition, previous work in this laboratory indicated a maximum energy absorption should occur at 1 MHz rather than the microwave frequencies typically employed.

Red blood cells will be exposed to CW and pulsed RF fields at several frequencies. The intention is to compare CW radiation at 1 MHz and 2450 MHz as well as to compare pulsed and CW fields at 2450 MHz for the same average power. Theoretical considerations suggest that at non-thermal average power densities in biological dielectrics radar type pulsed fields may induce ionic currents in the order of 1.06×10^{14} ions/cm² (7). Alternatively, CW exposures of long duration in thermoregulated chambers may also induce permeability alterations (8). In either case, parallel series will use the concentration of K⁺ and hemoglobin in the supernate to indicate changes in membrane permeability and hemolysis, respectively.

During FY 78, efforts were first directed at developing a thermoregulated exposure apparatus that provided a uniform field across the entire sample area. Other criteria were: (1) Operation at 1 MHz and 2450 MHz; (2) Good energy transfer to the blood; and (3) Input and output impedances close to 50 ohms.

A parallel plate waveguide configuration was selected and various shapes of blood holders inserted in the waveguide were evaluated using infrared thermography. A scheme using a dielectric ring around a small blood holder was found to work best.

Preliminary exposures were made of sheep red blood cells. During this time, blood handling and analysis procedures were worked out. Also, several mechanical problems in the exposure apparatus were revealed and corrected.

E. Neural Membrane Effects of Microwave Radiation (P.V. Brown, L.E. Larsen)

Various studies have suggested a fundamental interaction between excitable membranes and microwave energy which may not result from heating of the membrane (9). Microwave radiation of 12.5 cm wavelength at 11 mW/cm² CW for a duration of 30 min (2 °C heating) has been shown to increase conduction rates and shorten refractory periods in amphibian

peripheral nerve. Radiation with peak powers of 14 to 70 mW/cm² in pulses 1 ms. and PRR of 5/sec over a duration of 10 to 60 sec was found to alter the compound action potential. Similar results were obtained with 10 cm waves of higher power density (370 mW/cm² peak) in the form of 1 μ s pulses (PRR 700/s) where heating was limited to 0.2 °C. These and other findings suggest a fundamental interaction between excitable membranes and microwave radiation which may not be produced by "equivalent" thermalization.

Any microwave effects which are related to excitable membranes are especially relevant points of departure for a rational discussion of safety standards. Other investigators have used conventional metallic electrodes either in or outside the microwave field to record the action potential during or after microwave exposure. However, electrodes in the field distort and concentrate it, whereas electrodes outside of the field do not measure the properties of that part of the nerve exposed to microwave energy. An optical method is available to study excitable membranes. Specifically, the giant axon is known to display changes in the optical properties of the axoplasmic membrane which are directly related to the propagation of an action potential. In this way, membrane events are directly studied without the need for instrumentation within the biological preparation. It is these measures of membrane function which will be examined for evidence of microwave effects on axoplasmic membrane conducting an action potential. The question of induced field strength may be approached by IR pyrometry since the effective spot size can be as small as 175 μ m on an axon 500 μ m in diameter. Exposures take place in a transmission line where indirect measures of absorbed energy are possible. Although difficult to implement, the method allows one to study the part of the nerve exposed to microwave energy without distorting the field.

After last year, it was found that the giant axon from the Myxicola Infundibulum could not be used because the axon could not be completely cleaned of adhering muscle tissue without the use of enzymes which also affected electrical properties (10). Attempts were made to obtain giant axons from Lolliguncula Brevis squid, found year round in Florida. Unfortunately, these and other squid species survived only a few hours in the aquarium. Since the squid season was ending, it was decided to use a peripheral nerve from the crab. The crab nerve also would give a greater optical signal (100X) than the isolated axon. However, the nerve studies would not indicate the mechanism of interaction. That requires an isolated giant axon.

The instrument originally conceived to measure the optical change was found to be 100 times less sensitive than required. There was no way to increase its sensitivity enough, so another method for measuring the optical change was developed. Attempts to duplicate other workers' instruments were without success in spite of numerous consultations.

Other consultations were arranged, among them Lawrence Cohen. He was most helpful, lending us the necessary photodiode that was used with an internally developed ultra-low noise, high gain amplifier. Difficulties continued until TDY to Dr. Cohen's lab allowed us to find the problem in our technique.

We then successfully measured an optical change in the crab nerve that was time coincident with and the same shape as the action potential in the nerve. The S/N ratio was not good enough to identify changes due to microwave energy, so the equipment was further refined.

To detect the optical change in the nerve, one had to observe that part of the nerve adjacent to a stimulating electrode. Because the velocity of impulse propagation varied with the diameters of the axons that made up the nerve, the total nerve or compound action potential tended to "smear" as it propagated down the nerve. At a distance of more than 5 mm from the stimulating electrode, the optical change was reduced to the level of the background noise.

Solution of this problem required stimulation of the nerve in the microwave field with an optical path close to the stimulating electrode. A stimulating electrode was developed that was transparent to microwave energy. It employed RF compatible transmission lines from the MIC electrode program consisting of high resistance carbon loaded Teflon. Unfortunately, the use of this stimulating electrode reduced system sensitivity by limiting the available stimulus current to the nerve. Several further refinements to the lenses, light amplifier, and light source were made. Also, a new amplifier to record the nerve action potential through the high resistance lines was designed and built.

At this point, further improvement would have required purchasing a new microscope, lenses, and light source. Even though the S/N ratio was not too good, data was taken on three crab nerves exposed at 2450 MHz. The nerves were from different animals and each was exposed to a different power level, CW or pulsed energy. There was no microwave interference with equipment operation.

Future work will involve taking data at several different power levels and for greater number of nerves. The apparatus will be moved to a location more free of vibration and noise, which should improve the S/N ratio. Exposure to CW and pulsed microwave energy is planned. If a microwave induced change is found, the axon will be studied to determine the responsible mechanism.

F. Heat Loading and Conditioned Taste Aversion Learning in Rats (.G.R. Sessions)

This series of studies was designed to investigate the relationship between heat loading and conditioned taste aversion learning in rats, in an attempt to determine the degree to which microwave radiation is

uniquely responsible for the behavioral effects observed in an earlier microwave study.

Previously, this sensitive aversive conditioning paradigm was used to verify that the effects of microwave irradiation were sufficiently aversive to support this particular type of avoidance learning. The study also investigated the relationship between core temperature elevation, taste aversion learning, and incident microwave power density.

All irradiated groups showed a reliable decrease in preference for the normally desirable saccharin solution following five pairings with microwave exposure. However, in the 17 and 33 mW/cm² groups this effect was small, and there was no reliable correlation between power level and saccharin preference. The dose-response relationship was clearer at the highest power levels of 42 and 51 mW/cm², and the decreases in saccharin consumption were greater.

At 17 mW/cm² there was no reliable increase in colonic temperature following 30 minutes of radiation, but at power levels of 25 mW/cm² and higher, colonic temperatures increased monotonically between 1.5 and 3.0 degrees Celsius.

The present series of studies was designed to further investigate the relationship between core temperature elevation and taste aversion learning. It was reasoned that if avoidance conditioning could be achieved by elevating core temperature by environmental heating without microwave radiation, then a straight forward thermal interpretation of the earlier microwave-induced taste aversions would be warranted. On the other hand, if similar levels of avoidance conditioning cannot be achieved without microwaves, then some unique component of the microwave radiation would be implicated.

The results of this experiment showed that even though the temperatures of the high heat group reached 40-41° Celsius after the 30 minute heating, no evidence of taste aversion learning was observed in any of the postheating preference tests. The temperature elevations of the high heat group were almost identical to those obtained in the 42 mW/cm² groups in the microwave experiments. This group had shown significant taste aversion learning after microwave exposures.

A possible interpretation of the differences in results between the microwave and this heating experiment centers on the abrupt introduction of the animals into the heated chambers. The abrupt sensation of ambient heat elevation was an external stimulus which could have acted as an extraneous stimulus interfering with the association of subsequent heat stress with the previous saccharin consumption.

The same subjects from the first experiment were divided into three groups in a manner that counter-balanced the groups according to prior treatment. After several days of rehabilitation to the limited access drinking schedule, all animals were allowed 15 minutes access to a 2%

almond extract solution. They were then transported to environmental chambers as before. Two of these groups were then injected intraperitoneally with 30 mg/kg of cyclophosphamide, a drug known to be effective in inducing taste aversions in rats. One group, the heat-drug group, was exposed to high ambient heat (45° Celsius) for 4 minutes prior to these injections. The no heat-drug group received drug injections but no heat exposure. The third group served as saline-injected controls, half of which received the brief heat exposure. Three days after the conditioning trial all animals were tested for almond solution preference.

The preference tests showed that both drug-injected groups developed taste aversions to the almond solution. However, there was a slight decrement in the strength of the aversions in the heat exposed group, indicating that there was a slight interference with conditioning as a result of the brief heat exposure.

Another third experiment was conducted to determine if rats would develop taste aversions after heat exposure if the ambient temperature was gradually increased instead of abruptly raised. In addition, the heat interference issue was again addressed.

The results of the postheating preference testing showed that the group which was gradually heated without prior exposure showed slightly lower preferences for saccharin solution than both the abruptly exposed group and the controls. However, even though core temperature elevations were greater than those obtained with 42 mW/cm² microwaves, comparable taste aversion learning was not achieved.

Thus, in these experiments the external sensation of heat appears to interfere with the association of saccharin drinking and the aversive consequences of internal heat loading. Energy absorption in rats from a microwave field at 986 MHz is maximized considerably deeper than the skin, thus perhaps avoiding the external sensation of heat, and maximizing the aversive internal consequences of heat loading.

It can, therefore, be concluded from these experiments that the avoidance learning produced by microwave radiation in the earlier taste aversion experiments probably resulted from the unique properties of the microwaves, or at least from the unique way microwaves produce heat loading at high dose levels. It remains unclear whether or not non-thermal dose levels can produce conditioned taste aversions.

- G. Microwave Transparent MIC Electrodes for Temperature and Induced Electric Field Measurements (L.E. Larsen, R.A. Moore, J.H. Jacobi, P.V. Brown)

This project has seen three areas of development during the present report period building upon earlier published work (11). Two of these apply to the MIC temperature electrode. One was to explore methods of fabrication and feasibility of a totally flexible, thick film temperature

electrode. The intended use for such an MIC electrode was to permit intraluminal location of the device. This project resulted in the construction of several prototype electrodes, none of which performed as well as the previous hybrid thick/thin film electrode described in the 1977 Annual Report. The second project relevant to the MIC temperature electrode is to develop telemetry systems for use with the MIC electrodes within a microwave field. This project and its associated technologies are described further in the extramural segment, but it may be stated at this junction that the programmatic goal is to allow recording of temperatures and electric field induction without interposing a tether to the animal in the form of RF/MW compatible transmission lines.

Another area of electrode development was directed toward feasibility study and prototype construction for an electric field MIC electrode which could be implanted in biological dielectrics to provide in situ measurements of induced electric fields. This project produced prototype electrodes with good decoupling as evidenced by good thermographic appearance after exposure in tissue phantom dielectrics with a 250 mW/cm^2 , S band field. The electrode offers usable sensitivity in that a field density of 1 mW/cm^2 produced an output of 20 μV . In addition, the dimensions are compatible implantation insofar as the substrate is 0.025" square upon which are applied sputtered biconical dipoles, zero bias Schottky detector diode and thick film transmission line. Thus far, only uniaxial designs have been produced. These must be tested for interaction with the medium in which they operate. The next step will be the complete triaxial orthogonal design early in CY 79. This project, assuming continued good progress, will also be interfaced with the telemetry package. Details of the procedures and applications are proprietary and may not be publicly disclosed until DA patent action is complete.

H. High Speed Enzyme Inactivation In Situ by Microwave Energy Application (P.V. Brown, M.P. Toman)

In the present report period, the Microwave Research Department continued to support the Medical Neurosciences Department by further refinement of the modified Varian microwave source used for neurochemical analysis in rats and mice. The techniques developed in this department are the subject of a publication in IEEE Transactions on Biomedical Engineering which is abstracted below:

"The use of microwave energy to inactivate enzymes by rapid heating permits measurement of heat stable metabolites with minimal postmortem artifact. Reproducible results, however, require that the energy applied (dose) and the exposure geometry be reproducible. The commercial systems that we have examined are limited in this regard. A method to achieve a reproducible dose by electronic control of the applied power and exposure time is described. The electronics are compatible with any 220 volt, single phase commercial microwave power source. Without electronic control, exposure time varies by 50 milliseconds, due to random contactor

closure time. Output power varies by 15%, due to tube aging and variations in line voltage, magnetron temperature and load impedance during exposure. With the addition of electronic circuitry, the exposure time can be controlled within 3 milliseconds, and the power within 2%. Timing is done by counting energy bursts from the triac controlled magnetron. Leveling is accomplished using a feedback loop to vary the magnetron magnet current to keep the output power constant. The use of these electronic controls makes microwave irradiation systems more reliable as a biochemical research tool."

In addition, the microwave support engineer has been trained in the maintenance and operation of the inactivator. Also, an RFP was prepared for the construction of a higher powered and still more closely controlled inactivator with design principles based upon our experience with the Varian unit.

I. Viral Penetration

1. Studies of Electromagnetic Radiation as a Sensor and an Effector in Viral Penetration of Primate Cells In Vitro (L.E. Larsen, J.H. Jacobi, Dr. Berman, Dr. DuBois, M.P. Toman)

This project is a collaborative study with Drs. Berman and DuBois of the Department of Biologics, Division of Communicable Disease and Immunology. The objective is to explore the feasibility of using low power electromagnetic radiation to detect transmembrane transport of viral particles and to explore the feasibility of altering this penetration process with such radiation at higher field strengths. In addition, virus virulence may be affected by contiguous or prior exposure to electromagnetic radiation. The cell culture system then constitutes a biological amplifier of the effect to the extent that changes in viral multiplication will be mirrored by changes in culture plaquing. The project has proceeded to the step of designing an exposure apparatus which may be used with the biological system under study (cultured primate renal cells infected with denga virus), the measurement/calculation of field distributions in the chamber and measurement of temperature elevation in the culture medium. The project also required development of impedance transformation networks. These prevent further damage to the only 100 watt HF band power amplifier in the department. The delay occasioned by such damage prior to development of the networks prompted also the purchase of a back-up 10 watt amplifier (now over 6 months late in delivery).

We hope to overcome these pesty pragmatic problems and test the biological concepts by late in CY 78. The first experiments will be at essentially non-thermalizing field strengths, followed by thermalizing field strengths and conventional thermal controls.

J. Microwave Exposure Quality Assurance (M.P. Toman)

In collaboration with the Department of Medical Neurosciences, our department has prepared a set of performance requirements for an RFQ to construct a new microwave inactivator of thermolabile enzymes in situ. This project resulted in first round of bids, none of which were found to be suitable. The package was modified and new bids are to be received late in CY 78.

Two units were designed and tested for use in viral penetration studies in a collaborative study with Drs. Bermand and DuBois. These units employed predominantly electric field effects in one design and predominantly magnetic field effects in the other design. Preliminary data was collected concerning field strength, field patterns, etc. prior to delivery to Dr. Berman late in the present report period.

A temperature regulator was designed, built and tested for use with the parallel plate waveguide exposure system used for red blood cell permeability alterations due to electromagnetic radiation.

A revised and calculator based rather than computer based program for chamber calibration was developed.

K. Electronic Support (M.E.T. Swinnen)

An ultra-precise, linear voltage ramp, line driver and synchronous trigger pulse package was designed, constructed and tested to serve as input to the voltage controlled oscillator of the swept microwave source for the time delay spectrometer (TDS). The quality of this drive was sufficiently improved over commercially available signal generators that the spectral content of the TDS output more nearly approached monochromaticity, thereby improving differential propagation delay measurements.

A digital interface and buffer was designed, constructed and tested for the optical position encoders of the DART scanner. This project was necessary to allow completed computer control of the DART scanner under closed loop conditions.

Also, a 3-D isometric, graphic display for DART imagery data was designed and constructed. This display will be interfaced to the main processor/ controller to provide real time isometric display of the data with real time (hardware) multipliers for rotation of perspective, interactive depth cueing by intensity modulation, and hardware magnification/minification. This display will represent ca. \$6-7K in parts cost - yet equals the major features of systems costing ca. \$40-50K.

In addition, a number of smaller developments were undertaken for other investigators in the Division of Neuropsychiatry.

IV. Research Activities: Extramural Program

A. Measurement of Complex Permittivity of Biological Materials

This portion of the program took over funding from ARO for a project to develop a technique for in situ permittivity analysis in the range 100 MHz to 10 GHz. This project is based upon the concept that the impedance which represents the reflection coefficient of an antenna is a function of the complex permittivity of the medium into which the antenna radiates. This technique will offer in situ measurements which are capable of monitoring physiological responses to microwave exposure. The project began in the summer of 1978 and has been funded for two years. Present plans call for experiments to detect autolysis in both avascular and isolated organs.

A related project is field analysis within arbitrarily shaped, multilayered dielectrics exposed in waveguide. This project is directed toward exploring the effect of spatially varying permittivity on the uniformity of electric field induction in biological dielectrics. One practical application of this technique will be to provide analytical guidance for the development of improved microwave techniques for inactivational thermolabile enzyme (a collaborative project with Department of Medical Neurosciences). A new three dimensional field analysis technique was developed under this program which is based on the tensor Green's function. This analysis method was described in a publication in IEEE Transaction on Microwave Theory and Techniques.

The analytical results were subjected to intramural experimental verification by thermography of split block dielectric samples exposed in waveguide WR 284 at 2450 MHz. In the process, we discovered a discrepancy due to non-dominant modes. Recent recalculation of the interior fields failed to resolve the discrepancy. At this point, the project has already received three extensions, two at no cost. Further decisions await still another extension to explore higher order mode effects.

B. MIC Electrode Telemetry Package

A project plan has been developed and a contract was let via the RFQ mechanism for development of a telemetry package which can operate in microwave and UHF fields. The RFQ included miniaturization and micropower operation of the temperature encoding electronics described in the 1977 Annual Report. Details of the telemetry design are proprietary and may not be publicly disclosed until patent action is complete.

Project 3E162771A805 MICROWAVE INJURY

Work Unit 041 Biological effects and hazards of microwave radiation

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Project 3E162773A818

MEDICAL EFFECTS OF BLAST OVERPRESSURE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION		2. DATE OF SUMMARY		REPORT CONTROL SYMBOL	
				DA OC 6472		78 10 01		DD-DR&E(AR)636	
3. DATE PREV SUMMARY		4. KIND OF SUMMARY		5. SUMMARY SCTY		6. WORK SECURITY		7. REGRADING	
78 03 01		D Change		U		U		NA	
8. NO. / CODES		9. PROGRAM ELEMENT		10. PROJECT NUMBER		11. TASK AREA NUMBER		12. WORK UNIT NUMBER	
a. PRIMARY		62773A		3E162773A818		00		041	
b. CONTRIBUTING									
c. CONTRIBUTING									
13. TITLE (Precede with Security Classification Code)									
(U) Medical Effects of Blast Overpressure: Applied Studies									
14. SCIENTIFIC AND TECHNOLOGICAL AREAS									
017100 Weapons Effects 013300 Protective Equipment 00200 Acoustics									
15. START DATE				16. ESTIMATED COMPLETION DATE				17. FUNDING AGENCY	
78 03				Cont				DA	
18. CONTRACT/GRANT				19. PERFORMANCE METHOD				20. RESOURCES ESTIMATE	
a. DATES/EFFECTIVE: NA				b. EXPIRATION:				c. In-house	
d. NUMBER:				e. AMOUNT:				f. CUM. AMT.	
g. TYPE:				h. KIND OF AWARD:				i. PRECEDING	
j. KIND OF AWARD:				k. FISCAL YEAR				l. PROFESSIONAL MAN YRS	
m. KIND OF AWARD:				n. FISCAL YEAR				o. FUNDS (In thousands)	
p. KIND OF AWARD:				q. FISCAL YEAR				r. FUNDS (In thousands)	
s. KIND OF AWARD:				t. FISCAL YEAR				u. FUNDS (In thousands)	
v. KIND OF AWARD:				w. FISCAL YEAR				x. FUNDS (In thousands)	
y. KIND OF AWARD:				z. FISCAL YEAR				aa. FUNDS (In thousands)	
19. RESPONSIBLE DOD ORGANIZATION				20. PERFORMING ORGANIZATION				21. GENERAL USE	
NAME:				NAME:				22. KEYWORDS (Precede EACH with Security Classification Code)	
Walter Reed Army Institute of Research				Div of Med, Walter Reed Army Inst of Rsch				(U) Impulse noise; (U) Blast Overpressure;	
ADDRESS:				ADDRESS:				(U) Human Volunteer; (U) Temporary Threshold Shift; (U) Bioacoustics	
Washington, D.C. 20012				Washington, D.C. 20012				23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)	
RESPONSIBLE INDIVIDUAL				PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)				23. (U) To define the physiological effects upon the human of blast overpressure generated by firing Army weapons systems in terms of: (a) the physical characteristics of the pressure wave responsible for injury; (b) the interaction between the wave and susceptible organs; (c) the threshold for injury of the various organ systems and (d) potential means of prophylaxis and treatment of blast overpressure injury.	
NAME: ALTSTATT, Leslie B., COL, MC				NAME: SANDER, Gary E., MAJ, MC				24. (U) Procedures include the field measurement, recording, analysis and interpretation of the time-history and frequency content of the blast wave generated by firing large weapons, and audiometric evaluation of the temporary threshold shift induced in humans by such impulse noise. Under contract with the Lovelace Biomedical and Environmental Research Institute sheep have been exposed to a blast tube created pressure wave simulating the wave generated by field firing of the M198.	
TELEPHONE: (202) 576-3236				TELEPHONE: (202) 427-5041				25. (U) 78 03 - 78 09. Field measurements have been made on the M198, M109 and M110. The peak overpressures and B durations recorded have been applied to the criteria established in MIL-STD 1474 and firing limits derived for the M198 and M110. DH 178 helmets have been bench tested to determine the efficacy as acceptable acoustical muffs; the microphone circuitry was shown to produce significant distortion levels. In conjunction with Lovelace a waveform closely simulating the blast wave of the M198 with respect to peak pressure, A and B durations, and frequency content has been developed. Exposure of sheep to this simulated wave caused probable blast-induced lung injury at 185 dB and 190 dB peak pressure levels. JAYCOR corporation has been engaged to study the blast tube generated pressure wave and determine the degree to which it approximates the actual M198 wave, especially with regard to static and dynamic pressures. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 77 - 30 Sep 78.	
21. GENERAL USE				ASSOCIATE INVESTIGATORS				1 Oct 77 - 30 Sep 78.	
Foreign Intelligence Not Considered				NAME: PATTERSON, James, Ph.D.				* Available to contractors upon originator's approval.	
22. KEYWORDS (Precede EACH with Security Classification Code)				NAME:				DD FORM 1 MAR 68 1498	
(U) Impulse noise; (U) Blast Overpressure;				DA				PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.	
(U) Human Volunteer; (U) Temporary Threshold Shift; (U) Bioacoustics								1742	

Project 3E162773A818 MEDICAL EFFECTS OF BLAST OVERPRESSURE

Work Unit 041: Medical Effects of Blast Overpressure: Applied Studies

Investigators

Principal: MAJ Gary E. Sander, MC

Associates: Dr. James H. Patterson, Ph.D; CPT James Jaeger, MSC,
Dr. Marvin Stein, Ph.D., CPT Charles Burdick, MSC,
Ben Mozo, MS

Description

Studies are directed toward definition of the physiological effects upon the human of blast overpressure generated by firing Army weapons systems. Specific areas requiring investigation include: (1) the physical characteristics of the pressure wave responsible for injury; (2) the interaction between the blast wave and susceptible organs; (3) the threshold for injury of the various organ systems; and (4) potential means of prophylaxis and treatment of blast overpressure injury. In order to assess the potential of impulse noise to cause auditory and/or non-auditory injury, the impulse levels (overpressure levels) must be accurately recorded and analyzed and the results applied to MIL-STD 1474A (MI) to determine acceptable exposure limits. The instrumentation and methodology employed in making pressure measurements must be continually examined and if necessary modified to insure that all data essential to accurate health hazard assessment is available.

Progress

1. Background Information

Before discussing the state-of-the-art for auditory injury, certain terms must be precisely defined. Pressure levels are quantitated on two scales - either the PSI (pounds per square inch) scale which is linear, or the dB (decibel) scale, which is a logarithmic function of the PSI. It is of utmost importance to recognize that at pressures over 180 dB, the PSI scale increases rapidly relative to the dB scale. As an example, 180 dB corresponds to 2.8 PSI, and 183 dB to 4.2 PSI. An increase of only 1 dB may represent a significant pressure rise. The graphic representation of the peak pressure as a function of time is referred to as a time history. The peak pressure is the maximal pressure reached, the A-duration is the length of the initial pressure deflection, and the

B-duration the length of time until all pressure fluctuations cease. The frequency content is obtained by a Fourier transformation of the time history, and reflects the relative energy contributions of various frequencies. Blast overpressure and impulse noise are identical terms.

A general statement may be made about noise-induced hearing loss. Noise will cause hearing loss even at levels which will not cause eardrum rupture; adequate hearing protection will prevent this. What remains to be determined is the maximum noise levels for which adequate hearing protection can be provided. Single hearing protection consists of either an ear plug or an ear muff; double hearing protection consists of both modalities used together. Single protection provides about 25 dB noise attenuation at high frequencies; addition of the second modality provides an additional 5 dB attenuation for a total noise reduction of 30 dB. Both types of protectors are much less effective in attenuating low frequency noise; it is currently unresolved whether or not low frequency noise can induce high frequency hearing loss. The DH178 helmet is an "active muff." This is an ear muff modified by the addition of electronic circuitry such that it transmits and amplifies speech, but "cuts off" impulse noise. In itself it provides only single hearing protection; it must be used with ear plugs to provide double protection.

The current state-of-the-art document for use in estimating safe levels of noise exposure is the MIL-STD 1474A (MI) dated March 1975 (1). This document contains a damage risk criterion (DRC) for impulse noise which means that it attempts to assign a specific risk of injury for a given exposure. Once a particular blast wave is characterized by its peak pressure and B-duration, the position on the DRC may be determined and thus allowable exposures per 24 hours calculated. The "Z" line represents the maximum exposure level - even single exposures above this line are not permitted because hearing protection has not been demonstrated to be adequate at these levels and because of the possibility of non-auditory injury. Exposures under the "Z" line are permitted with double hearing protection; this limitation should protect 95% of individuals from incurring significant hearing loss. This DRC is derived directly from a CHABA (Committee on Hearing and Bioacoustics of the National Academy of Science - National Research Council) criterion published in 1968 (2). This criterion is based chiefly on empirical data from small arms fire and contains a number of clearly stated assumptions which have never been validated. These assumptions involve such important issues as the relationship between temporary (TTS) and permanent (PTS) hearing decrement and the manner in which it is measured, the way B-duration is defined, and that peak pressure and B-duration are the only important variables in determining injury.

Thus the MIL-STD is not the ultimate DRC for noise-induced injury, but it does represent the best existing document.

Canadian and British DRCs are also derived from the CHABA criterion, but are constructed such as to protect only 75% of exposed individuals. Hence they are less restrictive than the MIL-STD. The German DRC, promulgated by Prof. Pfander, does represent a significant departure from the MIL-STD (3). It is supported by a substantial body of data collected during actual troop exposure. A different definition of the time component is utilized as is a different approach to measuring temporary decrement. This DRC is more restrictive than the MIL-STD in certain situations, and less restrictive in others. A detailed comparison of the two is not possible with current data.

2. Requirements

There are two basic requirements which the auditory portion of the Blast Overpressure Program must address: (1) the accurate and reliable measurement of the pressure wave; and (2) the demonstration of the adequacy of hearing protection. At present, measurement of pressure waves is neither accurate nor reliable. Disagreement exists between USAARL and TECOM over the accuracy of overpressure measurements. Differences exist in the type of instrumentation utilized, the methods of calibration, and the procedure for data reduction. Demonstration of the adequacy of hearing protection will require the exposure of human subjects wearing hearing protection to gradually increasing pressure levels and careful monitoring to prevent excess temporary hearing decrements. Such data will be required to either validate the existing MIL-STD or to develop a more effective DRC.

3. Review of the Current Status of the Blast Overpressure Program

The auditory portion of this program is centered at USAARL, located at Ft. Rucker, Ala. Efforts have been primarily directed at field measurements of overpressures during M198 and M110 firings, bench testing of the DH 178 helmet, and preparation for a human volunteer study designed to test the double hearing protection concept.

Field measurements were made during the M198 firing at Yuma Proving Ground in April 1977 and during the M110 firing at Aberdeen Proving Ground in December 1977. Time histories were recorded at various crew positions for several weapon orientations and with the various charges. The time histories were then analyzed to determine peak pressures and B-durations and in some instances frequency contents. This data was then applied to the MIL-STD to derive those safety recommendations which will be discussed subse-

quently.

The DH 178 has undergone extensive bench testing to determine its performance as a passive noise attenuator (muff) and as an active circuit capable of transmitting and amplifying speech (4). The prototype helmet was demonstrated to offer good attenuation but to produce significant distortion of speech. As a result, of this testing and continuing interaction with Gentex (the DH 178 manufacturer), several modifications have been made in the electronics such that the DH 178 supplied for the M198 FOE offers both good noise attenuation and good speech transmission without distortion.

Evaluation of the overpressure data has pointed out several possible "shortcomings" in the methods of data collection. The transducers used for pressure recording are oriented in a "grazing" incidence (at right angles to the wave front); this orientation provides an accurate recording of the static component of pressure, but fails to record any dynamic component which the pressure wave may possess. The static component of pressure is that component which is the same in all directions; the dynamic component is produced by air moving with the wave front, and thus has direction. Animal studies which will be described subsequently have suggested that dynamic pressure may be important in producing lung injury, and yet the relative contribution of dynamic pressure to the total pressure in crew positions has not been measured. The methodology for measurement of dynamic pressure is presently being verified at USAARL using the small blast tube located there. It appears that by simultaneously recording with transducers oriented in both grazing and normal (directed toward the blast source) incidences the difference between the static pressure as recorded in grazing incidences and the pressure recorded in normal incidences represents the dynamic pressure.

MIL-STD 1474 currently specifies that pressure recordings be taken at approximate head location from the ground in positions where operators will stand. Since the chest is obviously located at a significantly lower level, and since the crewmen often crouch down during firing, the pressure to which the chest is exposed may be substantially higher than that pressure actually recorded. Thus it is critical to determine pressure variation as a function of height above the ground in the various operator positions. Such a three dimensional pressure map is referred to as a pressure contour map. Measurement of static and dynamic pressures and pressure contours will be made for the M198 in the near future.

The major USAARL time commitment during the past year has been directed toward a human volunteer study which would demonstrate the upper limits of overpressure for which double hearing protection is

adequate to prevent excess temporary hearing decrement in 95% of exposed individuals. This is accomplished by exposing subjects with initial normal hearing to gradually increasing overpressure levels and carefully monitoring these subjects audiometrically and adding hearing protection as required until excess temporary decrement occurs in subjects with double protection. Blast waves were to be simulated utilizing the blast tube facilities of the Lovelace Foundation. Since all changes in hearing acuity are temporary, no permanent hearing loss will occur in this study. A contract was negotiated with the University of New Mexico School of Medicine to provide volunteer subjects and audiometric monitoring. However, before the study could be undertaken, it was necessary to expose sheep to the blast wave to insure that no injury to non-auditory organs would occur. Thus because of the possibility of lung injury in the sheep, this study has been delayed until the question of injury in sheep can be definitively resolved.

Several contracts have been negotiated to support the auditory aspects of the Blast Overpressure Program. That contract with the University of New Mexico to provide human volunteers has been mentioned. Jaycor Corporation has been contracted to supplement USAARL in the field measurement and subsequent analysis of pressure data. Jaycor will initially assist USAARL in these tasks, and progressively take on greater responsibility as it gains experience. Jaycor will, in addition, perform a statistical analysis of the actual M198 generated blast wave and the blast tube simulated wave to determine the exactness of the simulation. A research proposal from John Erdreich, Ph.D., of the University of Oklahoma Health Sciences Center entitled "Studies of Aural Nonlinearity and the Mechanisms of Auditory Fatigue" has been funded. This investigator hopes to validate a simple audiometric procedure which will allow prescreening of individuals to determine those unusually sensitive and resistant to noise-induced hearing loss.

In this following section the interactions of the Blast Overpressure Program with the individual weapon systems will be described. The major effort has been directed toward M198/M203 due to the time constraints presented by the FOE now underway at Ft. Bragg, N.C. USAARL conducted extensive pressure measurements of the M198 with the M203, M3A1, and M4A2 charges at Yuma Proving Ground in April 1977. The final report with full analysis and interpretation of this data is now in final draft and will be published shortly. This data analysis demonstrates that overpressures generated during firing of the M203 charge exceed the Z-line at all elevations recorded - 45, 267, and 800 mil - in operator positions.

From this data two dimensional pressure contour maps can be constructed showing at what distance from the muzzle the Z-line

lies for each elevation at which the M203 was fired. Pressures are highest at 45 mil, somewhat less severe at 800 mil, and considerably lower at 267 mil. Thus barrel elevation is a most important parameter in determining peak pressures, with some protection afforded to operator positions at 267 mil. The reasons for such differences are not immediately obvious, but reflections from such weapon appendages as the trails seem to be one factor. By plotting the peak pressure as a function of distance from the muzzle along any radial it can be seen that the pressure increases as the wave crosses the weapon trail, apparently because of such a reflection from the trail. This phenomenon is most prominent at 45 mil, and is absent at 267 mil; there is a less intense but definitely present reflected wave at 800 mil. These contour maps indicate the complex nature of the pressure field and hence emphasize the importance of a detailed mapping of the pressure in operator positions, since relatively safer and less safe areas exist in close proximity. When potential reflecting surfaces are present the pressure at any given point is no longer a simple function of distance from the muzzle.

Utilizing these contour maps for the M203 charge, it can be determined that the Z-line is located approximately 20 feet to the rear of the breech. Thus in order to allow the M198 FOE to proceed on schedule, a number of recommendations were made such as to maintain operator exposures within the limits specified by the MIL-STD and hence to minimize if not eliminate all risk of auditory and non-auditory injury (5). These recommendations have been incorporated into the M198 Operators' Manual in use during the FOE. When firing the M203 charge, double ear protection must be worn (ear plugs and helmet with acoustical muffs), a 25 ft. lanyard must be used, no firing elevation less than 270 mil should be utilized, the crew stand sideways to the blast, and the firing be limited to 100 rounds per day. Furthermore, the maximum number of charges of other types with single protection and with double protection, respectively, is as follows: M3A1 - 251, and no limit; M4A2 - 16, and 160; M119 - 6, and 115. These restrictions apply if only a single charge is to be fired during a 24 hour period. In order to mix charges during a firing exercise, a point value may be assigned to each charge, and the point values added together until a point total of 1000 is reached for any 24 hr. period (6). The M203 is 10 points, the M119 - 9, M4A2 - 6, and the M3A1 - 1; any combination of charges may be utilized such that the total score remains under 1000. These recommendations are intended to apply solely to training situations, specifically the FOE. It is quite possible that they will be revised as more information becomes available.

USAARL and TECOM jointly instrumented and analyzed a M110 firing at APG in December 1977. This exercise stemmed from prior USAARL objections to certain TECOM overpressure data and was

directed at resolving this controversy. Analysis of the data collected by the two groups revealed that TECOM reported peak pressures lower than those measured by USAARL in 80% of instances; B-duration data was split randomly. The etiology of this peak pressure discrepancy is still under investigation, but there is a definite problem with baseline stability in at least one TECOM data collection channel. Unfortunately, the USAARL data could not be utilized to generate definitive firing guidelines in accordance with the MIL-STD because flash suppressant was erroneously omitted from all but the zone 9 charges. A large number of the time histories revealed a secondary blast peak resulting from muzzle flash which was higher in peak pressure than the initial peak, thus causing an artifactually high peak pressure. An USAARL representative monitored the TECOM instrumentation of a repeat M110 firing this time with all charges containing the flash suppressant; the unstable baseline is again present making all final data difficult if not impossible to interpret. A representative of the Blast Overpressure Program attended a M110 IPR dealing with the proposed ballistic shell; his recommendation that blast overpressure protection be made a design criterion was not accepted.

An extensive field measurement exercise with the M109/M203 is currently planned, but has been delayed until early 1979 by difficulties with the M203 charge. MRDC review of existing TECOM data with the M109/M203 reveals that overpressures in operator positions are well above the Z-line.

4. Impact of the Blast Overpressure Program Upon the Requirements

As discussed earlier, there are two basic requirements for the auditory portion of the program: (1) the accurate and reliable measurement of the pressure wave; and (2) the demonstration of the adequacy of hearing protection. In order to address the first requirement, USAARL is currently trying to resolve the issues of the relative contributions of the static and dynamic components to the total pressure wave and of the importance of microphone height. Measurements which will provide the necessary information are scheduled at Aberdeen from 29 Nov - 1 Dec. Such information will allow the construction of appropriate pressure contour maps to predict the safer areas for crew positioning around the M198. USAARL has also made some early progress in the demonstration of the adequacy of hearing protection. Extensive testing has been done with the DH178, resulting in substantial improvements in its electronic circuitry and providing an effective helmet/hearing protector for the M198 FOE. Preparations have been made for the human volunteer study to actually demonstrate the upper noise levels for which double hearing protection is adequate to protect hearing; this study will proceed as soon as safety from non-auditory injury can be

ensured. USAARL is also developing a basic research program studying mechanisms of hearing loss. The goal of the entire USAARL program is a methodology for predicting the attenuation offered by various protectors for a given pressure wave.

Project 3E162773A818 MEDICAL EFFECTS OF BLAST OVERPRESSURE

Work Unit 041 Medical Effects of Blast Overpressure: Applied Studies

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